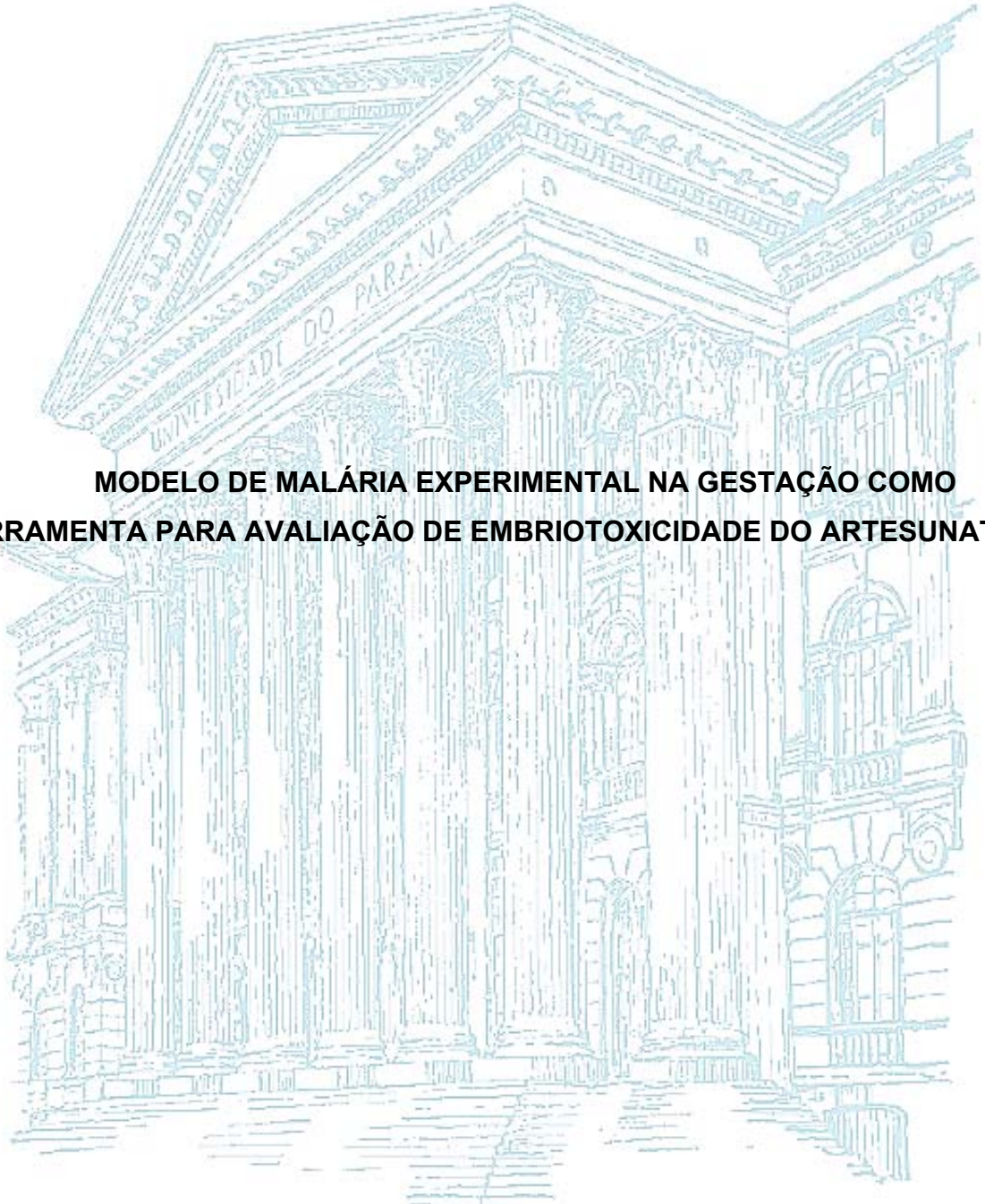
UNIVERSIDADE FEDERAL DO PARANÁ

CAROLINE GOMES



MODELO DE MALÁRIA EXPERIMENTAL NA GESTAÇÃO COMO FERRAMENTA PARA AVALIAÇÃO DE EMBRIOTOXICIDADE DO ARTESUNATO

CURITIBA

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MODELO DE MALÁRIA EXPERIMENTAL NA GESTAÇÃO COMO FERRAMENTA PARA AVALIAÇÃO DE EMBRIOTOXICIDADE DO ARTESUNATO

Tese apresentada como requisito parcial para obtenção do título de Doutor em Farmacologia, no Programa de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientador: Dr. Paulo Roberto Dalsenter

Coorientadora: Dra. Ana Cláudia Boareto

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **CAROLINE GOMES**, intitulada: "**MODELO DE MALÁRIA EXPERIMENTAL NA GESTAÇÃO COMO FERRAMENTA PARA AVALIAÇÃO DE EMBRIOTOXICIDADE DO ARTESUNATO**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

Curitiba, 19 de Abril de 2016.

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NOTA EXPLICATIVA

Esta tese é apresentada no formato de artigos para publicações de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. Neste formato constam: uma introdução, revisão de literatura, objetivos do trabalho, os artigos científicos, discussão e a conclusão geral da tese. O primeiro artigo é uma revisão de literatura e os dois artigos seguintes abordam os experimentos realizados, resultados e discussão. Os artigos foram formatados conforme as normas propostas pelos periódicos aos quais serão submetidos.

Aos meus pais, Rosa e Anísio (*in memorian*),
pelo amor e todos os valores transmitidos.

Aos meus irmãos, Dyego e Robson, e minha cunhada e irmã Andrea, que me completam e me inspiram.

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“Numa folha qualquer, eu desenho um navio de partida
com alguns bons amigos bebendo de bem com a vida.
De uma América a outra, consigo passar num segundo,
Giro um simples compasso e, num círculo, eu faço o mundo
Um menino caminha e caminhando chega no muro
e ali logo em frente, a esperar pela gente, o futuro está.”

Vinicius de Moraes e Toquinho

RESUMO

A malária é uma doença infecciosa responsável por milhares de mortes, principalmente em países tropicais. Crianças menores de cinco anos e gestantes são consideradas como grupos de alto risco, devido a maior susceptibilidade às formas severas desta infecção que podem ser letais. A malária durante a gestação pode causar abortos, parto prematuro, restrição no crescimento intrauterino, baixo peso ao nascimento e morte fetal. Devido a estes riscos para a mãe e para o feto as gestantes diagnosticadas devem ser tratadas imediatamente. Entretanto, para a maioria dos antimaláricos disponíveis não há dados consistentes que comprovem a segurança do seu uso durante a gestação. Atualmente, os derivados da artemisinina são os fármacos mais eficazes no tratamento da malária, porém devido à embrioletalidade e teratogenicidade observadas em estudos pré-clínicos o uso destes agentes durante o primeiro trimestre gestacional é restrito a casos de malária severa. Porém, até o momento, estes efeitos embriotóxicos não foram comprovados em humanos. Um dos diferenciais entre estudos clínicos e pré-clínicos é a presença da infecção. O mecanismo de ação dos derivados da artemisinina envolve certa seletividade aos parasitas e/ou eritrócitos infectados, deste modo seus efeitos tóxicos poderiam ser alterados em animais infectados. Assim, neste trabalho foram avaliados os efeitos embriotóxicos do artesunato em modelo de malária na gestação. Para isso, foi estabelecido um modelo de malária durante a gestação em camundongos suíços, onde animais infectados e não infectados foram expostos a diferentes doses de artesunato no dia 10 de gestação (dentro do período crítico de toxicidade), e posteriormente, seus efeitos sobre o desenvolvimento embrionário foram avaliados. O modelo estabelecido mimetizou características da malária durante a gestação em humanos, como maior susceptibilidade das fêmeas prenhas à infecção, parto prematuro, embrioletalidade, redução do peso fetal, retardo no desenvolvimento ósseo, danos na placenta e em múltiplos órgãos maternos. O tratamento com o artesunato na maior dose utilizada (30 mg/kg) demonstrou alta embrioletalidade e causou danos aos tecidos embrionários, corroborando dados da literatura. Foi verificada a eficácia do tratamento na redução da parasitemia e na prevenção dos efeitos negativos da infecção sobre o ganho de peso materno, peso de órgãos maternos e mortalidade embrionária. Houve uma redução na embrioletalidade, causada tanto pela infecção quanto pelo tratamento isoladamente, quando o artesunato na maior dose foi administrado em animais infectados. No entanto, ainda foram observados efeitos embriotóxicos do artesunato nesta dose, como redução de eritroblastos e danos histopatológicos nos embriões vivos de progenitoras infectadas e tratadas. É possível que tenha ocorrido uma redução na concentração do artesunato que chegou aos embriões devido à presença dos parasitas, no entanto este mecanismo deve ser investigado. Estes resultados demonstram a importância de considerar as alterações causadas pela presença da infecção no organismo hospedeiro na avaliação da toxicidade de antimaláricos. Neste sentido, o modelo aqui estabelecido terá grande aplicabilidade em estudos futuros. Contudo, neste estudo pré-clínico os benefícios do tratamento com o artesunato foram superiores aos riscos da infecção sobre o desenvolvimento embrionário.

Palavra-chave: modelo de malária experimental; malária placentária; derivado da artemisinina; artesunato; embriotoxicidade; embrioletalidade, teratogenicidade.

ABSTRACT

Malaria is an infectious disease responsible for thousands of deaths, mainly in tropical countries. Children under five and pregnant women are considered as groups at high risk, due to their higher susceptibility to the severe forms of this infection which can be lethal. Malaria during pregnancy can cause miscarriage, preterm delivery, intrauterine growth restriction, low birth weight and fetal death. Due to these risks for both the mother and the fetus, diagnosed pregnant women must be promptly treated. However, for the majority of antimalarial drugs available there are no consistent data to prove the safety of their use during pregnancy. Currently, artemisinin derivatives are the most effective drugs for malaria treatment, but due to embryolethality and teratogenicity observed in non-clinical studies the use of these agents during the first trimester of pregnancy is restricted to cases of severe malaria. Nevertheless, until now these embryo toxic effects have not been proved in humans. One of the differences between clinical and non-clinical studies is the presence of the infection. The mechanism of action of artemisinin derivatives involves a certain selectivity to parasites and/or infected erythrocytes, so their toxic effects might be altered in infected animals. Thus, in this work, the embryo toxic effects of artesunate were assessed in a malaria model during pregnancy. For this, a malaria model during pregnancy was set in Swiss mice, in which uninfected and infected animals were exposed to different doses of artesunate in gestational day 10 (within the critical toxicity period), and thereafter, its effects on embryo development were assessed. The model set mimic features of malaria during pregnancy in humans, such as higher susceptibility of pregnant females to infection, preterm delivery, embryolethality, reduction on fetal weight, delay in bone development, placental damage and alterations in multiple maternal organs. The treatment with artesunate in the highest dose used (30 mg/kg) showed high embryolethality and caused damage to embryonic tissues corroborating literature data. It was verified the efficacy of the treatment in the reduction of parasitemia and preventing the negative effects of the infection on maternal weight gain, maternal organs weight, and embryonic mortality. There was a reduction on embryolethality, caused by either the infection or the treatment isolated, when the artesunate in the highest dose was administered to infected females. However, the embryo toxic effects of artesunate were still seen on this dose, as like a reduction of erythroblasts and histopathological damage in live embryos from females infected and treated. It is possible to have occurred a reduction in the artesunate concentration which reaches the embryos due to the presence of parasites, but this mechanism needs to be investigated. These findings show the importance to consider the alterations caused by the presence of the infection in the host organism in evaluating the toxicity of antimalarial drugs. Therefore, the model set here has great applicability in future studies. Withal, in this non-clinical study, the benefits of treatment with artesunate were higher than the risks of the infection on embryo development.

Keywords: experimental malaria model; placental malaria; artemisinin derivative; artesunate; embryotoxicity; embryolethality, teratogenicity.

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ACTs - do inglês *Artemisinin-based combination therapies*
IPTp - do inglês *Intermittent preventive treatment in pregnancy*
MS - Ministério da saúde
OMS - Organização Mundial da Saúde;

Artigo científico 1

ACTs - Artemisinin-based combination therapies
DHA - dihydroartemisinin
FADH - flavin adenine dinucleotide
GD - gestational day
HIF-1α - hypoxia-inducible factor-1α
ROS - reactive oxygen species
VEGF - vascular endothelial growth factor
WHO - World Health Organization

Artigo científico 2

CYP - cytochrome P450 enzymes
GD - gestational day
GFP - green fluorescent protein
IEs - infected erythrocytes
IL-6 - Interleucina-6
IL-10 - Interleucina-10
IUGR - intrauterine growth retardation
TNF-α - Fator de necrose tumoral

Artigo científico 3

ACTs - Artemisinin-based combination therapies
CNS - central nervous system
GD - gestational day
PNS - peripheral nervous system
RBC - red blood cells
WHO - World Health Organization

SUMÁRIO

1. INTRODUÇÃO

A malária permanece como um grave problema de saúde mundial responsável por grande morbidade e mortalidade. Esta doença infecciosa é causada por parasitas do gênero *Plasmodium* e transmitida por mosquitos vetores do gênero *Anopheles*, sendo que a forma mais grave da doença é causada pelo *P. falciparum* (WHO, 2015a).

Estima-se que anualmente mais de três bilhões de pessoas estão expostas ao risco de contrair esta infecção (WHO, 2015a). No Brasil são registrados mais de 100.000 casos por ano, dos quais cerca de 90% são causados por *P. vivax* (MS, 2015a).

A sintomatologia da malária varia de acordo com a espécie infectante, parasitemia e grau de imunidade do hospedeiro. Crianças menores de 5 anos e gestantes são mais susceptíveis aos efeitos graves da malária que podem levar à morte (MS, 2010).

As mulheres que são infectadas durante a gestação apresentam maiores riscos de desenvolver complicações graves como a anemia severa e malária cerebral (DESAI *et al.*, 2007). Abortos, parto prematuro, baixo peso ao nascimento e mortalidade infantil são alguns dos efeitos negativos da malária durante a gestação (UMBERS *et al.*, 2011).

Devido a estes riscos, a Organização Mundial da Saúde (OMS) recomenda a quimioprofilaxia para gestantes que vivem em áreas de alta intensidade de transmissão. O diagnóstico e tratamento imediato dos casos são essenciais para redução da transmissão e da mortalidade associadas à malária (WHO, 2015b).

Atualmente existem poucos fármacos disponíveis para o tratamento da malária, principalmente devido ao desenvolvimento de resistência pelos parasitas. Na década de 70 foi isolada da planta *Artemisia annua* L. a substância antimalárica artemisinina. Posteriormente, foram desenvolvidos derivados semissintéticos de maior potência. Devido à alta eficácia e ação rápida a combinação de derivados da artemisinina com outros antimaláricos tornou-se uma das primeiras linhas de tratamento da malária. Estas drogas tiveram grande importância na redução dos casos desta doença observada nos últimos anos (WHITE *et al.*, 2015; WHO, 2015b).

Tal descoberta salvou milhares de vidas e conferiu à farmacologista chinesa Youyou Tu o prêmio Nobel de fisiologia e medicina no ano passado (WHITE *et al.*, 2015).

As opções terapêuticas para o tratamento da malária durante a gestação são ainda mais escassas. A segurança do uso de antimaláricos durante este período crítico do desenvolvimento é pouco avaliada e para a maioria dos antimaláricos não há dados consistentes quanto aos possíveis riscos ao feto. Atualmente a OMS recomenda o uso de quinina e clindamicina na manutenção de casos de malária *falciparum* durante o primeiro trimestre gestacional. Nos demais trimestres é aconselhado o uso de cloroquina ou combinações de antimaláricos contendo derivados da artemisinina, variando de acordo com a espécie infectante ou a severidade dos casos (WHO, 2015b).

Os derivados da artemisinina são considerados seguros e eficazes, porém a segurança do seu uso durante o primeiro trimestre gestacional não está bem estabelecida. As dúvidas recaem sobre estudos pré-clínicos de toxicidade reprodutiva que demonstraram letalidade embrionária e teratogenicidade em várias espécies (CLARK *et al.*, 2004; LONGO *et al.*, 2006; CLARK *et al.,* 2008; WHITE e CLARK, 2008; BOARETO *et al.*, 2012, 2013). Todos os derivados da artemisinina são considerados toxicantes do desenvolvimento em animais por mecanismos de toxicidade não completamente esclarecidos.

Até o momento, tais efeitos tóxicos ao embrião não foram evidenciados em humanos. Existem poucos estudos clínicos com pequeno número amostral e por isso baixo poder estatístico para detectar uma incidência maior de abortos ou efeitos teratogênicos do que a encontrada na população em geral (WHO, 2015b).

Nesse sentido, um dos diferenciais entre estudos clínicos e pré-clínicos com os derivados da artemisinina na gestação é a presença de alterações no organismo hospedeiro causadas pela infecção. Modelos de malária experimental na gestação têm sido utilizados para elucidação dos mecanismos fisiopatológicos desta doença (VAN ZON e ELING, 1980; VAN ZON *et al*., 1985; NERES *et al.*, 2008; MARINHO *et al.*, 2009; BARBOZA *et al.*, 2014). Considerando que a fisiopatologia da malária envolve interações que ocorrem entre o parasita e o hospedeiro, tais modelos apresentam-se como ferramentas úteis para estudos farmacológicos e toxicológicos.

Assim, neste estudo avaliamos a toxicidade embrionária do artesunato, um dos derivados da artemisinina mais utilizados (WHO, 2015b), em animais infectados

com *P. berghei* ANKA. Foram avaliados os efeitos da infeção e do artesunato, isolados e associados, sobre o desenvolvimento embrionário em modelo de malária experimental durante a gestação utilizando camundongos suíços.

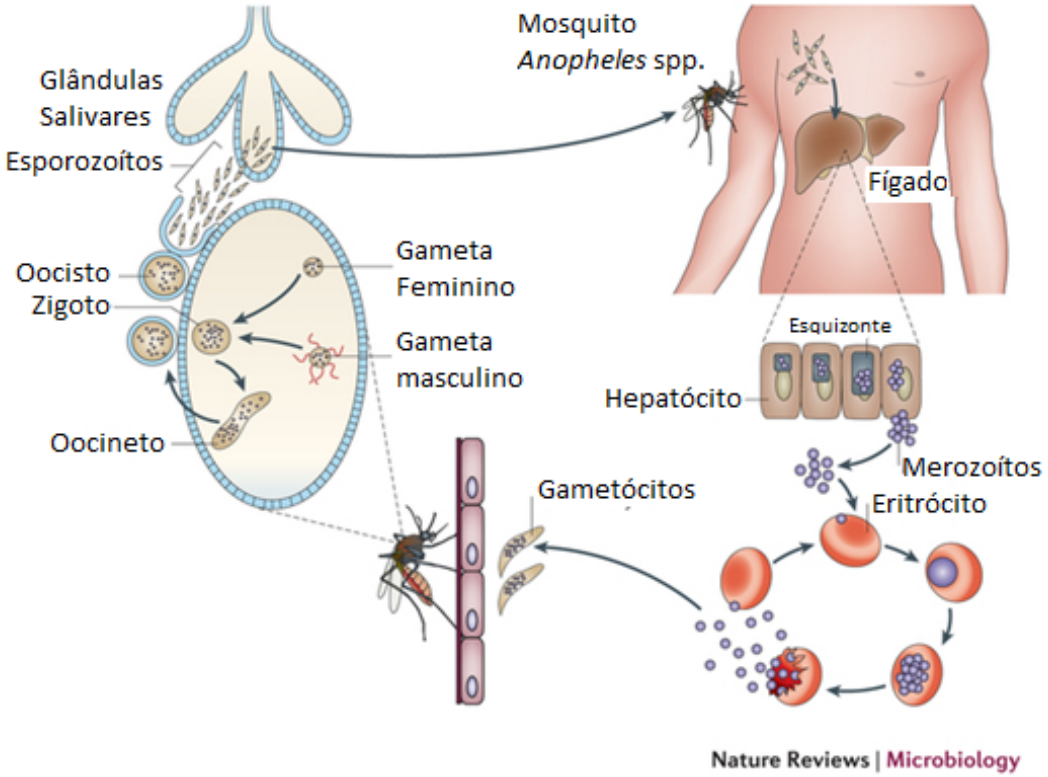
2. REVISÃO BIBLIOGRÁFICA

2.1 MALÁRIA

A malária é uma importante doença infecciosa, causada por protozoários intracelulares do gênero *Plasmodium* e transmitida por mosquitos fêmeas do gênero *Anopheles*. Independentemente da espécie de plasmódio, quando não tratada, esta doença febril aguda pode evoluir para as formas severas e levar a morte. Das cinco espécies infectantes para os humanos o *P. falciparum* é o que apresenta maior severidade, sendo o responsável pela maioria dos casos letais (WHO, 2015b).

Quando um mosquito infectado faz o repasto sanguíneo em um indivíduo são inoculadas as formas esporozoítas do parasita que primeiramente irão se multiplicar no fígado, caracterizando a fase hepática da doença (FIGURA 1). Após este período serão formados os merozoítos que atingirão a corrente sanguínea infectando as hemácias, esta fase é denominada de fase sanguínea. Os parasitas irão se multiplicar assexuadamente no interior das hemácias formando esquizontes que irão romper e liberar diversos merozoítos para infectar novas hemácias. Por vezes, serão formados os gametócitos, as formas sexuadas do parasita, que são as formas infectantes para o mosquito. Os gametócitos irão se reproduzir no interior do mosquito e formar novos esporozoítos perpetuando o ciclo biológico do *Plasmodium* (FLANNERY *et al.*, 2013).

FIGURA 1 - CICLO BIOLÓGICO DO *Plasmodium* spp.



FONTE: Adaptado de Flannery e colaboradores 2013.

Na fase sanguínea é que são observados os sinais clínicos da malária. Os sintomas costumam aparecer 10 a 15 dias após a infecção e inicialmente são muito inespecíficos, como febre alta, calafrios, vômitos e cefaleias. No entanto, uma característica são os picos de febre em intervalos regulares de tempo de acordo com a espécie de *Plasmodium*, de 48 a 72 horas, relacionados aos ciclos sanguíneos dos parasitas (MS, 2010).

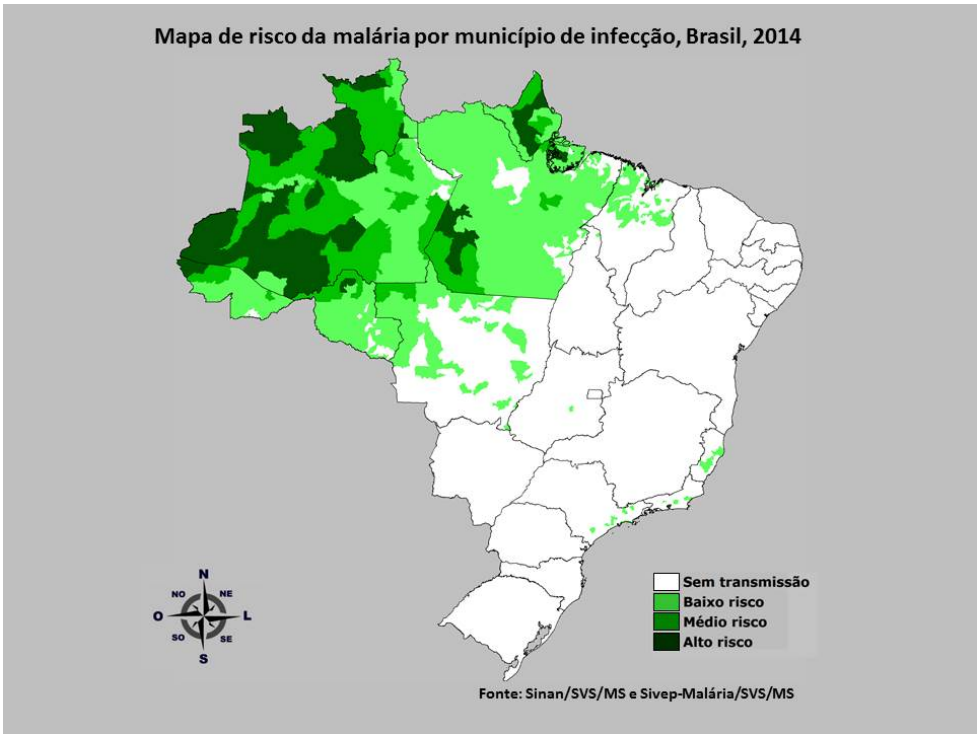
Esta doença permanece como uma importante causa de morbidade nos países tropicais e subtropicais, onde 96 países tem transmissão contínua da mesma. A intensidade de transmissão depende da associação de fatores do ambiente, dos vetores, do parasita e do hospedeiro. Geralmente os mosquitos do gênero *Anopheles* tem o hábito de alimentação noturno (WHO, 2015b).

Estimativas da OMS relatam que quase metade da população mundial está exposta ao risco de contrair malária. São registrados anualmente cerca de 200 milhões de casos e mais de 400 mil óbitos em consequência desta doença (WHO, 2015b).

No Brasil a malária é considerada endêmica na região amazônica. Esta área engloba os estados do Acre, Amapá, Amazonas, Pará, Rondônia, Roraima,

Tocantins, e parte do Mato Grosso e do Maranhão (FIGURA 2). Esta região possui fatores climáticos e socioeconômicos que favorecem a transmissão da doença. Diversas estratégias vêm sendo realizadas a fim de controlar a transmissão da malária no Brasil (OLIVEIRA-FERREIRA *et al.*, 2010).

FIGURA 2 - MAPA DAS REGIÕES DE RISCO DE TRANSMISSÃO DA MALÁRIA NO BRASIL EM 2014



FONTE: MS, 2015b.

A ação de programas de luta contra a malária tem resultado em uma redução expressiva do número de casos da doença e também de mortes. De acordo com OMS entre 2000 e 2015 ocorreu uma redução de 37% na taxa de incidência e de 48% na taxa de mortalidade por malária a nível mundial. Estas reduções se devem em grande parte ao uso de mosquiteiros impregnados com inseticidas e da terapia combinada com derivados da artemisinina (ACTs - do inglês *Artemisinin-based combination therapies*). No entanto, milhões de pessoas ainda não têm acesso às estratégias de contenção da doença (WHO, 2015a).

As gestantes e as crianças menores de 5 anos são considerados como grupos de alto risco e como tal requerem especial atenção (WHO, 2015a). Na África, onde ocorrem cerca de 90% das mortes, especialmente na região subsaariana, estima-se que uma criança morra a cada minuto devido à malária (WHO, 2015c).

2.2 MALÁRIA DURANTE A GESTAÇÃO

Estima-se que em 2007, aproximadamente 125 milhões de gestantes estiveram expostas ao risco de contrair malária (DELLICOUR *et al.*, 2010). Quando ocorre durante a gestação a malária pode causar efeitos adversos tanto à mãe quanto ao feto. Tais efeitos irão depender da espécie infectante, características imunológicas do hospedeiro, tempo de gestação e densidade de transmissão da malária na região (HVIID *et al.*, 2010).

Durante a gestação ocorrem mudanças hormonais e imunológicas para o desenvolvimento fetal normal. O sistema imune é desviado de uma resposta inflamatória para uma resposta anti-inflamatória. Isto se justifica basicamente pelo fato de que o feto tem antígenos paternos e pode ocorrer a rejeição do mesmo quando há prevalência de uma resposta inflamatória. Por outro lado, essa transição da resposta imunológica pode alterar a patogênese de doenças infecciosas (ROBINSON E KLEIN, 2012). Esta imunossupressão materna foi à explicação inicial para a maior susceptibilidade das gestantes à malária (MENENDEZ et al., 1995; NOSTEN *et al*.,2004). No entanto, estudos mais recentes têm demonstrado que a placenta tem um papel central na patogênese da malária na gestação (HVIID *et al.*, 2010).

A severidade da malária causada pelo *P. falciparum* é atribuída à adesão de eritrócitos parasitados por esta espécie às células endoteliais da microvasculatura de vários órgãos do hospedeiro, como o cérebro, resultando em malária cerebral. Isto ocorre devido à expressão de proteínas nos eritrócitos parasitados que se ligam a receptores específicos presentes nas células do hospedeiro. Na placenta ocorre a grande ligação destes eritrócitos ao receptor de Condroitina Sulfato A (CSA) nos espaços intervilosos, resultando em amplo sequestro de eritrócitos parasitados neste tecido e selecionando uma subpopulação de parasitas que se ligam ao CSA (FRIED e DUFFY, 1996, FRIED *et al*., 2006).

A placenta se apresenta com um reservatório de eritrócitos parasitados ocasionando em um mecanismo de evasão do parasita da circulação periférica o que prejudica a sua destruição pelo baço (ROWE e KYES, 2004; GREENWOOD *et al*., 2008). Também ocorre uma resposta inflamatória (SUGUITAN *et al*., 2003) e deposição de material fibrinóide na placenta (WALTER, GARIN e BLOT, 1982), o

que pode resultar na redução do fluxo sanguíneo placentário (DORMAN *et al*., 2002) e, subsequentemente, ao baixo peso ao nascimento e prematuridade (MENENDEZ *et al*., 2000; BRABIN *et al*., 2004).

Em áreas de baixa transmissão sabe-se que as gestantes apresentam maior susceptibilidade à infecção em relação a mulheres não gestantes ou homens. As gestantes têm maior probabilidade de desenvolver anemia severa, malária cerebral e outras síndromes da malária severa, assim como podem ocorrer abortos, parto prematuro, baixo peso ao nascimento e natimortos (DESAI *et al.*, 2007; HVIID *et al.*, 2010).

As infecções durante a gestação nestas áreas cursam com considerável parasitemia e sintomatologia principalmente devido à baixa imunidade adquirida. A paridade (número de gestações) é um fator de menor importância nestas áreas onde a grande maioria das gestantes apresenta alto risco de complicações em consequência da malária (BRABIN *et al.*, 2004; DESAI *et al.*, 2007).

Em áreas de alta transmissão as gestantes que contraem malária são geralmente assintomáticas embora a carga parasitária seja maior que em não gestantes. Ao contrário das áreas de baixa intensidade de transmissão a paridade tem um grande efeito sobre a gravidade da infecção nestas áreas (HVIID *et al.*, 2010). Na primeira gestação há maior risco de bebês com baixo peso ao nascimento do que nas gestações subsequentes (DESAI *et al.*, 2007; DELLICOUR *et al.*, 2010). Esta proteção à malária placentária e ao baixo peso fetal nas gestações subsequentes está associada à produção de anticorpos que bloqueiam a ligação de eritrócitos infectados ao CSA por gestantes que contraem malária (FRIED *et al*., 1998; DUFFY e FRIED, 2003).

O baixo peso ao nascimento é sem dúvida um dos grandes efeitos adversos da malária na gestação e está associado a um aumento da mortalidade infantil (DELLICOUR *et al.*, 2010; UMBERS *et al.*, 2011). Na África subsaariana a malária gestacional é responsável por cerca de 20% dos casos de baixo peso ao nascimento (DESAI *et al.*, 2007).

O baixo peso ao nascimento pode ocorrer como consequência tanto de um retardo no crescimento intrauterino quanto do parto prematuro. O retardo no crescimento intrauterino parece ser o fator mais importante em áreas de alta transmissão, enquanto que o parto prematuro em áreas de baixa transmissão. É

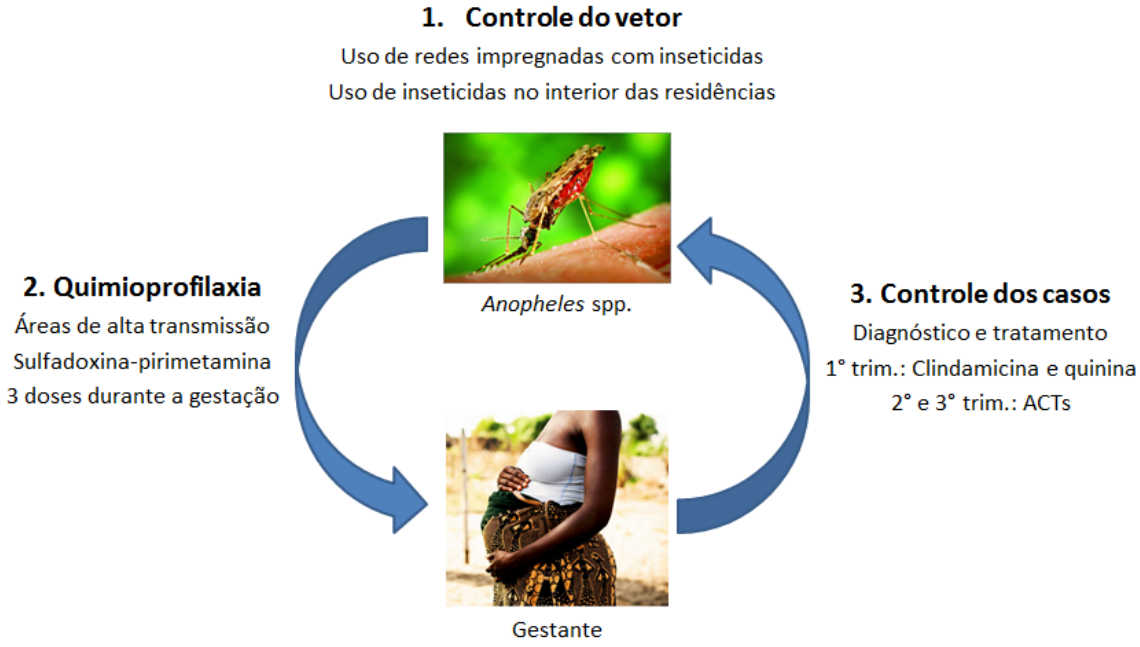
provável que isto esteja relacionado ao fato de que a imunidade adquirida previne episódios febris e anemia severa que podem levar ao parto prematuro (DESAI *et al.*, 2007).

Entretanto, é importante ressaltar que em ambos os casos, tanto no parto prematuro quanto no retardo do crescimento intrauterino, o baixo peso ao nascimento é um fator que aumenta a mortalidade infantil, sendo a malária durante a gestação uma condição que contribui indiretamente para tal efeito adverso (LUXEMBURGER *et al.*, 2001).

2.3 PREVENÇÃO E TRATAMENTO DA MALÁRIA NA GESTAÇÃO

Estratégias de controle da malária na gestação incluem o uso de redes impregnadas com inseticidas para proteção contra os mosquitos, aplicação de inseticidas no interior das residências, quimioprofilaxia, diagnóstico e tratamento (FIGURA 3). O acesso a tais estratégias tem crescido consideravelmente nos últimos anos e contribuído para redução no número de casos. No ano de 2014 foram distribuídos cerca de 190 milhões de mosquiteiros na África, no entanto, este número ainda está aquém do ideal para proporcionar a todos os indivíduos em risco este controle primário do vetor (WHO, 2015b).

FIGURA 3 - PRINCIPAIS ESTRATÉGIAS DE PREVENÇÃO E TRATAMENTO DA MALÁRIA NA GESTAÇÃO



Os tratamentos indicados são para casos de malária não complicada causada por *P. falciparum*.
FONTE: esquema modificado e adaptado de WHO (2015a), foto do mosquito vetor (GATHANY, 2015) e foto da gestante (LSTM, 2013).

Em áreas de alta transmissão é recomendado pela OMS estratégias de quimioprofilaxia aos grupos de risco, como gestantes e crianças menores de cinco anos. Nestas áreas o tratamento intermitente preventivo (IPTp - do inglês *Intermittent preventive treatment in pregnancy*) com sulfadoxina-pirimetamina deve ser administrado a todas as gestantes nas duas primeiras gestações iniciando no segundo trimestre gestacional. Quase todos os países da África subsaariana, onde a transmissão é contínua, adotaram o IPTp. Entretanto, o percentual de gestantes que recebeu ao menos uma dose deste tratamento preventivo foi de 52% em 2014, e menor ainda para às que completaram as três doses recomendadas durante a gestação (WHO, 2015b).

O diagnóstico e tratamento imediato são essenciais para redução dos riscos associados à malária. O tratamento tanto profilático quanto terapêutico são imprescindíveis devido aos riscos da infecção para a mãe e para o feto. Atualmente é preconizado o uso de quinina e clindamicina no primeiro trimestre gestacional para o tratamento da malária não complicada causada por *P. falciparum* ou caso não seja confirmado a espécie infectante. No primeiro trimestre gestacional é recomendado o

tratamento da malária não complicada causada por outras espécies o uso de cloroquina ou quinina. Em ambas as situações o uso de ACTs é recomendado no segundo e terceiro trimestres. O uso do artesunato ou do artemeter, ambos derivados da artemisinina, seguido por ACTs é permitido para o tratamento da malária severa no primeiro trimestre gestacional devido ao risco de morte materna ou fetal (WHO, 2015b).

No Brasil, são seguidas as recomendações da OMS para a prevenção e tratamento da malária. Não é adotado o IPTp devido a menor proporção de casos de malária severa por *P. falciparum*. Porém todas as demais estratégias de prevenção e tratamento são seguidas. Isto inclui a distribuição de mosquiteiros impregnados com pesticidas piretroides, recomendações de uso de repelentes e demais medidas de proteção individual a todos os grupos de risco (MS, 2010).

A terapêutica com derivados da artemisinina foi adotada como primeira escolha para o tratamento de malária causada por *P. falciparum* a partir de 2006 no Brasil. Isto contribuiu para a grande redução no número de casos observados nos anos subsequentes (OLIVEIRA-FERREIRA *et al.*, 2010). O artesunato e o artemeter são os derivados da artemisinina mais utilizados (MS, 2010).

Contudo, as estratégias de prevenção e tratamento da malária na gestação são escassas e restritas. Existem poucos dados sobre a segurança do uso de antimaláricos na gestação (WELLS *et al.*, 2015). Atualmente a OMS considera seguros para uso no primeiro trimestre gestacional quinina, cloroquina, clindamicina e proguanil. A emergência de resistência à maioria dos antimaláricos disponíveis, incluindo aos derivados da artemisinina é preocupante. Neste cenário é evidente a necessidade de desenvolvimento de novas terapias especialmente para os grupos de maior risco, como gestantes (WHO, 2015b).

2.4 MODELOS DE MALÁRIA NA GESTAÇÃO

Compreender a patogênese da malária durante a gestação é importante para o desenvolvimento de novas estratégias terapêuticas. Parasitas do gênero *Plasmodium* que infectam roedores têm sido amplamente utilizados para a compreensão desta patologia (HVIID *et al.*, 2010; ZUZARTE-LUIS *et al.*, 2014).

Modelos de malária na gestação foram desenvolvidos utilizando cepas de *P. berghei* ou *P. chabaudi* em combinação com diferentes linhagens de camundongos hospedeiros (VAN ZON e ELING, 1980; VAN ZON *et al*., 1985; HVIID *et al.*, 2010; ZUZARTE-LUIS *et al.*, 2014).

Neres e colaboradores (2008) utilizando camundongos BALB/c infectados com o *P. berghei* ANKA evidenciaram características da malária placentária causada por *P. falciparum* em mulheres grávidas. Neste trabalho as fêmeas prenhas foram infectadas pela via intravenosa com 10^6 eritrócitos parasitados no 13° dia de gestação. Foi demonstrada maior susceptibilidade à malária nas fêmeas prenhas, redução no número de fetos viáveis, redução do crescimento intrauterino, aumento no número de abortos, alterações placentárias, aderência de eritrócitos infectados na placenta e retardo no crescimento dos filhotes nascidos vivos (NERES *et al.*, 2008).

A infecção de camundongos C57BL/6 com *P. berghei* NK65, K173 ou ANKAΔpm4 no mesmo dia gestacional do estudo citado acima também promoveu alterações histológicas e inflamação placentária afetando negativamente a viabilidade fetal (RODRIGUES-DUARTE *et al.*, 2012).

Outro modelo experimental mimetizou regiões de alta transmissão de malária onde as mulheres adquirem certa imunidade antes de engravidar (HVIID *et al.*, 2010). Neste modelo as fêmeas foram infectadas e tratadas anteriormente aos cruzamentos sendo observado a recrudescência da infeção durante a primeira, segunda e terceira gestações. A taxa de recrudescência e os prejuízos observados em decorrência da infecção foram gradualmente menores conforme o número de gestações (MARINHO *et al.*, 2009; MEGNEKOU *et al.*, 2009, 2013). Este modelo tem grande utilidade para a compreensão dos mecanismos imunológicos envolvidos na malária durante a gestação.

No entanto, poucos estudos avaliaram os efeitos de drogas antimaláricas em modelos experimentais durante a gestação. O perfil farmacocinético do tratamento com a quinina no final da gestação foi avaliado em fêmeas prenhas infectadas com *P. berghei* (LIRUSSI E PUSSARD, 2006). Foi demonstrado um aumento na concentração plasmática de quinina em fêmeas prenhas infectadas em relação às prenhas não infectadas. No entanto, as concentrações de quinina na placenta, no feto e líquido amniótico apresentaram-se reduzidas devido à infecção. Isto evidencia

que alterações em órgãos do hospedeiro devido à malária podem modificar a farmacocinética das drogas e a sua distribuição para os compartimentos feto-placentários.

Sharma e Shukla (2014) avaliaram os efeitos do tratamento antimalárico preventivo com cloroquina ou sulfadoxina-pirimetamina em um modelo de malária experimental na gestação. Foi verificado um aumento no estresse oxidativo na placenta devido à infecção e à redução deste efeito pelo tratamento. Todos os filhotes de fêmeas infectadas morreram em até 30 dias após o nascimento, o tratamento preventivo foi capaz de evitar esta mortalidade pós-natal. Um desequilíbrio dos níveis de antioxidantes e oxidantes e o envolvimento de apoptose via mitocondrial na patologia da malária placentária foi previamente descrito pelo mesmo grupo (SHARMA *et al.*, 2012a,b).

Apesar dos estudos supracitados, até o momento não foram avaliados os efeitos de tratamentos antimaláricos, após o estabelecimento da infecção, sobre o desenvolvimento embrionário em modelo de malária experimental.

3. HIPÓTESES E PREDIÇÕES

Nossa hipótese é de que a malária possa alterar os efeitos embriotóxicos do artesunato observados em animais não infectados. Da mesma maneira, nos propusemos a avaliar os efeitos da infecção isoladamente sobre o desenvolvimento embrionário e os possíveis benefícios do tratamento. Caso esta hipótese seja verdadeira, esperamos verificar alterações significativas nas análises de embriões de grupos infectados e tratados com artesunato quando comparados àqueles que receberam apenas o antimalárico.

4. OBJETIVOS

4.1 OBJETIVO GERAL

- Avaliar os efeitos embriotóxicos do artesunato em modelo de malária na gestação.

4.2 OBJETIVOS ESPECÍFICOS

- Padronizar um modelo de malária na gestação em camundongos suíços, estabelecendo um dia de infeção que possibilite o tratamento com o artesunato no 10° dia gestacional (dia mais sensível para teratogenicidade do artesunato).
- Investigar os efeitos da malária sobre o desenvolvimento esquelético de fetos expostos *in utero* à infecção.
- Avaliar os efeitos da infeção sobre o ganho de peso gestacional e peso de órgãos maternos.
- Verificar possíveis alterações histopatológicas na placenta características da malária no modelo estabelecido.
- Avaliar os efeitos do tratamento com artesunato em fêmeas prenhas infectadas e não infectadas sobre o ganho de peso gestacional e peso de órgãos maternos.
- Avaliar os efeitos do tratamento com artesunato sobre o desenvolvimento embrionário em embriões expostos ou não à infecção através de análises histopatológicas.

5. ARTIGOS CIENTÍFICOS

5.1 ARTIGO 1: CLINICAL AND NON-CLINICAL SAFETY OF ARTEMISININ DERIVATIVES IN PREGNANCY

Este trabalho consiste de uma revisão bibliográfica sobre o uso dos derivados da artemisinina na gestação. Este artigo foi aceito para publicação no periódico *Reproductive toxicology*, como requisito para defesa de doutorado.

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Clinical and non-clinical safety of artemisinin derivatives in pregnancy

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Highlights

- Artemisinin derivatives are embryo toxic to uninfected experimental animals.
- No embryotoxicity was proved in clinical studies until now.
- There are species differences in the estimated sensitive period for toxicity.
- The presence of malaria infection might reduce the distribution of drug to fetus.
- Further researches are needed to clarify the developmental toxicity of these drugs.

Abstract

Malaria in pregnancy is a clinically wasting infectious disease, where drug therapy has to be promptly initiated. Currently, the treatment of this infection depends on the use of artemisinin derivatives. The World Health Organization does not recommend the use of these drugs in the first trimester of pregnancy due to non-clinical findings that have shown embryolethality and teratogenic effects. Nevertheless, until now, this toxicity has not been proved in humans. Artemisinin derivatives mechanisms of embryotoxicity are related to depletion of circulating embryonic primitive erythroblasts. Species differences in this sensitive period for toxicity and the presence of malaria infection, which could reduce drug distribution to the fetus, are significant to the risk assessment of artemisinin derivatives treatment to pregnant women. In this review we aimed to assess the results of non-clinical and clinical studies with artemisinin derivatives, their mechanisms of embryotoxicity and discuss the safety of their use during pregnancy.

Keywords: artemisinin derivative; embryotoxicity; clinical study; non-clinical study; pregnancy.

1. Introduction

Malaria is a severe infectious disease that remains a major public health challenge in endemic regions, including countries from South and Central America, Africa and Asia. This disease is caused by parasites of the *Plasmodium* genus and

transmitted by *Anopheles* mosquitoes. The most severe form of malaria is triggered by *P. falciparum*. It is estimated that annually more than 3 billion people are at risk of contracting malaria and 400,000 deaths are recorded as a result of this disease [1].

During pregnancy, malaria can be a clinically wasting condition. Its complications are remarkable in pregnant women, such as severe anemia and cerebral malaria, and their offspring face possibility of stillbirths, miscarriages or low birth weight [2]. Antimalarial drug therapy during pregnancy has to be promptly initiated, making the safety of currently available drugs and their combination for mothers and their babies a research topic of paramount relevance. Moreover, due to the risk of malaria to the mother and the fetus, the World Health Organization (WHO) recommends chemoprophylaxis for pregnant women living in high-intensity transmission areas [3]. Nevertheless, based on non-clinical data, there are restrictions about malaria treatment for pregnant women, especially in the first trimester [4].

Currently, there are few drugs available for the treatment of malaria. Parasites, particularly *P. falciparum*, have become resistant to conventional drugs [5]. In the 70's, the antimalarial substance artemisinin was isolated from *Artemisia annua* L. (figure 1a). Subsequently, higher power semi-synthetic derivatives of artemisinin were developed. Due to the high efficacy and rapid action of artemisinin derivatives in combination with other antimalarial agents, they have become one of the first line treatments for malaria. These drugs have had great importance in reducing the incidence of malaria [1, 6]. This discovery has saved thousands of lives and rewarded the Chinese pharmacologist Youyou Tu the Nobel Prize for Physiology and Medicine in the last year [6].

The therapeutic options for the treatment of malaria during pregnancy are even scarcer. In the second and third trimesters of pregnancy, or in severe cases, the use of antimalarial combinations containing artemisinin derivatives is advised [3]. The WHO recommends the use of quinine and clindamycin in the first trimester, because the safety of artemisinin derivatives has not been well established in this period. Concerns were raised in non-clinical studies that have shown embryonic lethality and teratogenicity in several species [7-9]. However, such toxicity has not been proved in humans [3].

This review intends to provide data on developmental toxicity of artemisinin derivatives and discuss their safety during pregnancy in clinical and non-clinical trials, as well as discuss their mechanisms of embryotoxicity.

2. Artemisinin and its derivatives

Artemisinin is extracted from the leaves of the *A. annua* L. (figure 1a), and has been used in China for 2000 years as an antipyretic, referred to as *qinghao* [10, 11]. It was discovered, purified and identified in 1972 [12]. Artemisinin is a sesquiterpene lactone containing an endoperoxide bridge, which is believed to be necessary for antimalarial activity (figure 1) [13]. Artemisinin is an extremely active antimalarial to treat uncomplicated and severe malaria [6].

The artemisinin content in dried leaves is between 0.06 to 2% and it is poorly soluble in water or oil [14]. The artemisinin low bioavaibility and poor pharmacokinetics properties are the major drawback of its use. After its discovery, several semisynthetic derivatives were identified including the dihydroartemisinin (DHA) which is more potent and chemically stable than artemisinin (figure 1b) [15]. Water-

soluble (artesunate and artelinate) and oil-soluble (artemether and arteether) artemisinin derivatives were synthetized and developed (figure 1b), and they are known as first generation endoperoxides [14]. After administration, these derivatives are metabolized in DHA (figure 1c). The antimalarial effect of the artemisinin derivatives results primarily from DHA, which disappears from plasma with a half-life of approximately one hour [16]. Artemisinin derivatives are currently the most important class of antimalarial drugs [3].

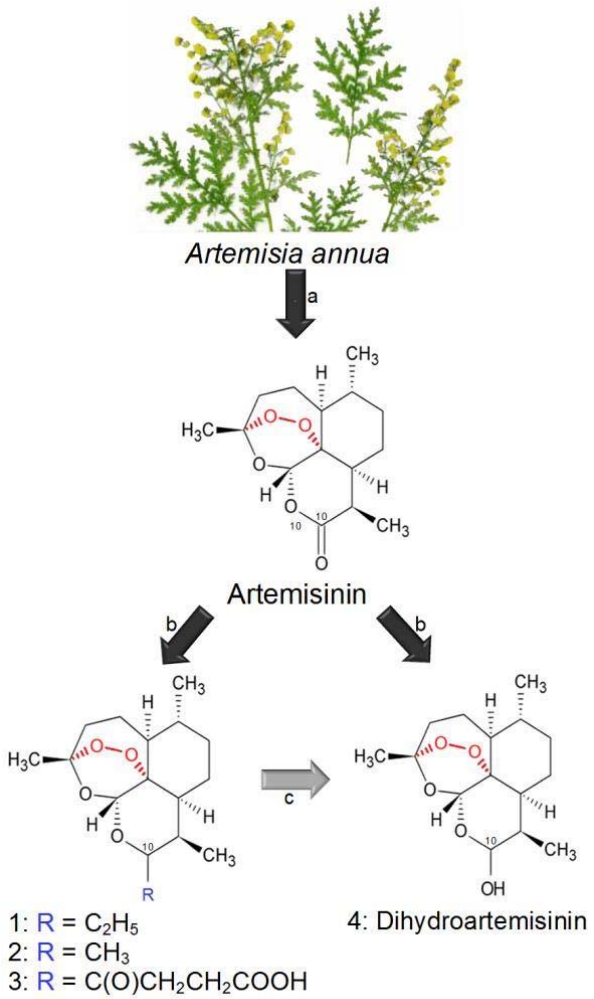

Figure 1: Chemical structures of artemisinin (ART) and some of its derivatives. ART is extracted from *Artemisia annua* (**a**) [6]. Different compounds can be synthetically (**b**) obtained from ART by chemical modifications at C10 position (**R**=radical), such as arteether (**1**), artemether (**2**), artesunate (**3**) and dihydroartemisinin (DHA) (**4**) [17]. The ART derivatives **1-3** are converted *in vivo* (**c**) into DHA (**4**), which has higher antimalarial activity than ART and contributes significantly to the antimalarial activity of these drugs [15, 16]. Artemisinin and its derivatives have an endoperoxide bridge (stressed in red), which is the pharmacophore for their activity.

Efforts to find more metabolically stable artemisinin derivatives are ongoing and have highlighted the second generation of endoperoxides, including 10-(alkylamino)-artemisinins, as artemisone and artemiside [10]. They are called second generation because they are more stable and potent than the others derivatives [14]. As only endoperoxide bridge is required for antimalarial activity, significant efforts have been focused on identification of fully synthetic artemisinin-like peroxides with a simple and cheaper synthesis, as for example trioxanes and diterpenes peroxides with antimalarial activity. However, these newly developed semi-synthetic and synthetic derivatives are still undergoing development [10].

Artemisinin and derivatives are rapidly effective and well tolerated but, due to the fact they have short half-life, monotherapy is not recommended to avoid resistance [6, 11, 14]. Therefore, artemisinin derivatives are administered combined with others antimalarial to increase efficacy and adherence to the treatment [6, 18]. The use of artemisinin-based combination therapies (ACTs) for uncomplicated malaria has enabled a reduction of treatment from 7 (artemisinin monotherapy) to 3 days, avoiding recrudescence [3, 6].

The most common ACTs used for malaria treatment are artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine and artesunate-sufadoxine-pyrimethamine [10, 19]. ACTs for treatment of *falciparum* malaria reduce load gametocytes, reducing retransmission, but this effect is incomplete without the inclusion of primaquine, which is a known gametocytocide [3].

The exact mechanism of the antimalarial activity of the artemisinin derivatives remains controversial. Within the malaria parasitized erythrocyte, hemoglobin is degraded by a series of protease enzymes of parasite to release peptides and amino acids required for its development and to create space within its digestive vacuole,

increasing the amount of free iron and heme [20]. One possible mechanism of action suggests that artemisinin derivatives, into the parasitized erythrocyte, accumulate and release free radicals through loss of endoperoxide bridge by iron or heme, which kills the parasite [21]. Recently, Wang et al. [22] showed that heme, rather than free ferrous iron, is predominantly responsible for artemisinin activation.

The artemisinin activation generates carbon-centered radicals which are highly reactive and can covalently bind to several proteins and alkylate them impairing their function [20]. These radicals alkylate heme and form heme-drug adducts which have been verified by *in vitro* and *in vivo* studies [23-26]. This nonpolymerizable heme may accumulate and produce toxic reduced oxygen species being toxic to the malaria parasites [27]. Furthermore, it was demonstrated that artemisinin can covalently bind to more than one hundred parasite proteins, many of these proteins are important for parasite survival, such as a known artemisinin target P*f*ATP6, a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) enzyme [21, 22].

Artemisinin derivatives kill the intraerythrocytic forms, and their action on the young stages of *Plasmodium* prevents the development of pathological complications [10]. The artemisinin derivatives are more toxic to parasitized erythrocyte, which absorbs better the drug, being 100 times more concentrated in infected erythrocytes as compared to uninfected erythrocytes [22, 28].

Currently, malaria therapy depends on the use of artemisinin derivatives and on the ACTs. In Southeast Asia, development of artemisinin-resistant strains of *P. falciparum* put at risk the benefits obtained by the reduction of malaria mortality achieved with the inclusion of this treatment since the year 2000; however, these drugs are still the best choice for *falciparum* malaria [3]. Additionally, artemisinin derivatives are being investigated as anticancer agents as well because they inhibit

angiogenesis and cell growth in several neoplastic cell models [29]. These compounds have also demonstrated efficacy in the treatment of schistosomiasis and fasciolasis and in animal models of *Clonorchis* infection [30, 31].

3. Non-clinical developmental toxicity studies with artemisinin derivatives

Since artemisinin derivatives were discovered and emerged as highly effective antimalarial to treat multidrug resistant parasites, their developmental toxicity has been evaluated. Initial studies conducted in mice, rat, rabbit, guinea pig and hamster demonstrated embryolethality, resorptions and whole litter resorption after administration of artemether, artesunate and DHA by different routes and range of doses between gestational days (GD) 5 to 18 [32, 33]. Malformations were first reported by Li (1988) [34] who evidenced umbilical hernia and rib defects on fetuses after parenteral administration to pregnant rats of 26 mg/kg of artesunate from GD 6 to GD 8 (cited by Clark et al. [7]).

Many groups have described teratogenic effects of artemisinin derivatives in animals as mainly cardiovascular and skeletal defects, which happen in association with increased resorptions (Table 1). Malformations on cardiovascular system consist in wall heart, ventricular septal and great vessel defects. The skeletal malformations include long bones, scapulae and ribs defects. These drugs seem to be more embryo-lethal than teratogenic and the doses that induce these effects were close to each other in rats and rabbits [7].

The WHO concludes that all artemisinin derivatives were developmental toxicants showing embryolethality or teratogenicity in animals [35]. Embryo toxic

effects of these antimalarial drugs have been subsequently confirmed in different species, including monkeys (Table 1).

These embryo toxic effects, as well as their antimalarial activity, seem to be a “class effect” depending on the presence of endoperoxide bridge [36]. However, arterolane (also called OZ277), a fully synthetic artemisinin derivative in phase III of clinical trials [37], showed a better safety margin than artemisinin and DHA in a comparative study using rat whole embryo culture (WEC) [36]. This brings light to the possibility of keeping apart antimalarial activity from embryo toxic effect.

Artemisinin derivatives monotherapy is not allowed due to emergence of resistance (WHO, 2015). A lot of ACTs have been produced as fixed-dose combination to reduce monotherapy inadequate use [3]. Besides that, non-clinical reproductive toxicity studies with ACTs are very scanty (Table 2). The combination of artesunate-dapsone-chlorproguanil showed classic embryo effects attributable to artesunate treatment alone with no additive effect to its toxicity [7]. Interestingly, Boareto and co-workers showed that artesunate and mefloquine co-administration reduced embryo toxic effects of artesunate treatment alone by mechanisms not yet elucidated [9, 38]. It makes evident the importance to assess ACTs potential of developmental toxicity. This could help finding less harmful options for the treatment of malaria during pregnancy.

Additionally, long-term effects on offspring after prenatal antimalarial treatment with artemisinin derivatives were also poorly evaluated. Our group evaluated the effects of *in utero* exposure to oral artemisinin treatment (7 mg/kg - GD 14-20) to rats and evidenced a reduction on sperm count and daily sperm production on male offspring and did not find any effect on female offspring [39]. Changes on sperm count were previously mentioned by WHO and raised concerns about the

interference with male reproductive system [35]. A recent study also reported a reduction on sperm number and viability in adult rats exposed to 35 mg/kg of artemisinin for 7 days accompanied by testes and epididymis histopathological alterations [40]. The effects of these drugs on reproductive system remains to be better evaluated.

Table 1. Non-clinical developmental toxicity studies of artemisinin derivatives isolated treatment

Drug	Species	Route	Dosage (mg/kg/day)	Period of treatment	Embryo/fetal viability	Developmental toxicity
AS	Rat	Oral	6 to 16.7	GD 7-19	Increased post-implantation losses (including total litter loss)	Reduced fetal body weight, skeletal and cardiovascular defects [7].
AS	Rabbit	Oral	5 to 12	GD 6-17	Increased post-implantation losses at 12 mg/kg	Skeletal and cardiovascular defects on highest dosage [7].
DHA	Rat	*In vitro* WEC	0.01 to 2 µg/mL	GD 9.5 to 11.5	No effect on embryo viability	Exposure for 48 hours at 0.05 µg/mL and above led to pale yolk sac, reduced number of erythroblast and inhibition of angiogenesis underlying embryo anatomical abnormalities and cell death [41].
DHA	Rat	Oral	7.5 and 15	GD 9.5 and 10.5	Increased embryo death on GD 13-20	Mainly skeletal and cardiovascular malformations in surviving fetuses from litters with embryo deaths (C-section on GD 20). Cell death areas and erythroblasts alterations on embryos histological examination before GD 20 [8].
AS	Rat	Oral	17	GD 10 or 11	77% of embryo death on GD 14	Pale yolk sac, reduction in crown-rump length, erythroblasts depletion and heart abnormalities [42].
ATM	Rat	IP	1.5; 7 and 15	GD 0-7	No alterations	No effects on fetal body weight and growth rate of offspring until 5 weeks post-natal [43].
AS	Rat	Oral	10,17 and 30	GD 9-16 single or multiple days	Embryolethality was seen after treatment with single or multiple doses on GD 10-14	GD 10-14 was the sensitive period for cardiovascular malformations and skeletal defects. GD 10 was the most sensitive day for these teratogenic effects. These malformations appeared on litters with partial resorption [44].
ART	Rat	Oral	7, 35 and 70	GD 7-13 GD 14-20	100% of embryolethality at two highest doses on GD 7-13, and at 70 mg/kg on GD14-20.	It was demonstrated a reduction on sperm number on male offspring after *in utero* exposure to 7 mg/kg/day on GD 14-20 [39].
DHA	Frog	*In vitro* FETAX	0.01 to 0.5 µg/mL	24 to 72 or 120 to 168 hpf	No effect on embryo viability	Exposure for 48 hours above 0.05 µg/mL showed pale heart with less primitive red blood cells inside and at the highest concentrations (0.1 and 0.5 µg/mL) major abnormalities, mainly in the heart, have occurred [45].

AS: artesunate; GD: gestational day; DHA: dihydroartemisinin; WEC: whole embryo culture; ATM: artemether; IP: intraperitoneal; ART: artemisinin; FETAX: Frog embryo teratogenesis assay-xenopus; hpf: hours post-fertilization; ATE: arteether; AA: artelinic acid; AA-EE: *A. annua* ethanolic extract.

Table 1. Continued

Drug	Species	Route	Dosage (mg/kg/day)	Period of treatment	Embryo/fetal viability	Developmental toxicity
AS	Monkey	Oral	4, 12 and 30	GD 20-50	Embryolethality was seen at 12 mg/kg and 30 mg/kg after 12 days of treatment	Surviving embryos showed reduced erythroblasts number, cardiomyopathy and slight reduction on long bones length [46].
AS DHA ATM ATE	Rat	Oral	15 AS 11.1 DHA 19.4 ATM 20.3 ATE	GD 10	Embryolethality	It was observed the same pattern of developmental toxicity for all derivatives: cardiovascular and skeletal abnormalities [47].
ATM	Rat	Oral	3.5 and 7	GD 0-6 GD 7-14 GD 14-20	100% resorptions at 7 mg/kg administrated on GD 7-14	Reduction on fetal body weight and retarded skeletal development but no skeletal malformations were reported when the treatment was in the organogenesis period [48].
AS ATM ATE	Rat	IP	10/20 AS 8/16 ATM 15/30 ATE	GD 6-15	Whole litter resorption at higher doses	Fetuses exposed to AS and ATE (10 and 15 mg/kg respectively) presented shortened bones (humerus, femur, tibia, fibula) and a reduction on fetal weight, crown-rump and tail length [49].
AS AA	Rat	Oral	35/48 AS 17.2-191 AA µmol/kg	GD 12	Embryolethality at higher doses	No external alterations [50].
AS	Rat	Oral	15 and 40	GD 9-11	Embryolethality including whole litter resorptions at 30 mg/kg	Higher incidence of deficient ossification and skeletal malformations, mainly abnormalities on limb long bones and scapulae [38].
AS	Rat	Oral	15 and 40	GD 9-11	Embryolethality	Reduced erythroblasts, pale yolk sac and histopathological abnormalities on embryos mainly on liver and heart [9].
AS	Rat	Oral	2, 4 and 8	GD 6-15	Increased post-implantation losses at 8 mg/kg	Increased visceral and skeletal variations at 4 and 8 mg/kg and reduced female fetal weight at 8 mg/kg [51].
AA-EE	Rat	Oral	100, 200 and 300	GD 8-19	The highest dose reduced fetal viability	External malformations were observed at highest dose [52].

The studies are presented in chronological order.
AS: artesunate; GD: gestational day; DHA: dihydroartemisinin; WEC: whole embryo culture; ATM: artemether; IP: intraperitoneal; ART: artemisinin; FETAX: Frog embryo teratogenesis assay-xenopus; hpf: hours post-fertilization; ATE: arteether; AA: artelinic acid; AA-EE: *A. annua* ethanolic extract.

Table 2. Non-clinical developmental toxicity studies of artemisinin-based combination therapies (ACTs)

ACT	Species	Dosage (mg/kg/day)	Period of treatment	Embryo/fetal viability	Developmental toxicity
AS-CP- DP	Rabbit	4.7 to 11.8 - AS 2.4 to 5.9 – CP 2.9 to 7.4 – DP	GD 7-19	Increased postimplantation losses (including total litter loss)	Reduced fetal body (mid and high dose), skeletal and cardiovascular defects attributed to artesunate [7].
AS-CP-DP	Rat	5.9 to 23.5 – AS 2.9 to 11.8 – CP 3.7 to 14.7 – DP	GD 6-17	Increased postimplantation losses at 23,5 mg/kg/day (including total litter loss)	Reduced fetal body weight, skeletal and cardiovascular defects at highest dosage attributed to artesunate [7].
AS-MQ	Rat	15 and 40 – AS 30 and 80 – MQ	GD 9-11	Embryolethality related to AS and reduced when combined with MQ	Limb long bone malformations related to AS treatment which was reduced when administrated the combination [38].
AS-MQ	Rat	15 and 40 – AS 30 and 80 – MQ	GD 9-11	Embryolethality related to AS and reduced when combined with MQ	Histopathological abnormalities on embryos including cell death areas and reduction on erythroblasts for AS alone or combined with MQ. Co-administration reduces these AS effects [9].

The studies are presented in chronological order.
All studies used oral route of administration. GD: gestational day; AS: artesunate; MQ: mefloquine; CP: chlorproguanil; DP: dapsone.

4. Clinical studies in pregnancy with artemisinin derivatives

It is difficult to compile data from clinical studies because of the huge heterogeneity of methodologies, different dose regimens and standard protocols for - treatment of severe or uncomplicated malaria. Many pregnant women are not aware about their own pregnancy and are inadvertently exposed to ACTs to treat malaria episodes, and the miscarriages, which happen especially in this period, may not be detected. The majority of studies are with several ACTs but there were a few studies with monotherapy or both. Another problem is the absence of precise information about gestational time of exposure and different ways to define that. Moreover, the endemic malarial regions have other socio-economic issues, such as the prevalence of many other disorders during pregnancy and a precarious health attendance [53]. Most of clinical trials with antimalarial drugs exclude pregnant women, which makes it difficult to identify adverse effects in this group [54].

The use of artemisinin derivatives is allowed for treatment of malaria during pregnancy in the second and third trimesters. Clinical studies have demonstrated that the use of ACTs in the second and third trimesters is safe for both pregnant women and their babies [55-57]. Over 4000 pregnancies did not show any adverse effects when exposed to these drugs in those trimesters [3]. The same safety information is provided by non-clinical studies on late gestation [44].

The lack of clinical studies and the results from animal studies led WHO to recommend that ACTs not be used in the first trimester of pregnancy [54]. However, the use of artemisinin derivatives, mainly artesunate, is allowed to treat severe malaria in the first trimester due to high rates of mortality, stillbirth and miscarriage [3]. The effective treatment of malaria during pregnancy is evidenced by the

prevention of low birth weight caused by this infection [55]. The available information about artemisinin derivatives exposure in the first trimester of pregnancy is in briefed on Table 3.

To this date, no studies in humans can relate exposure to artemisinin derivatives to increased risk of miscarriage, stillbirth and congenital anomalies as summarized on table 3. In view of these data, there are a few subjects exposed to these drugs in the first trimester. Some adverse effects are reported, such as miscarriage or a few congenital abnormalities, which are not clearly caused by ACTs. These effects are usually correlated with malaria infection or their incidences are not higher than local population rates. It suggests that more studies are required to verify the safety of ACTs in the first trimester of pregnancy.

Table 3. Effects of artemisinin derivatives or ACTs for treatment of malaria in the first trimester of pregnancy in women

Drug	Route	Total dose[a]	Exposure	Pregnant women	Effects observed
AS	Oral	840 mg	Treatment of recrudescent infections – inadvertently exposed (GW 3-12)	15	There were 20% (3/15) of spontaneous abortion, which was not considered different from the rate in the general population. 8 followed babies were normal until one year of life [58].
AS-PSD	Oral	200 mg AS Single dose	Preventive (Accidentally)	77	There were 5% of babies exposed (of 119 in any trimester) with physical abnormalities after birth, including umbilical hernia and undescended testis, but this was not statistically different from unexposed fetuses. No abortions, stillbirths or infant deaths were related to treatment [59].
AS[b] ATM[b] AS-MQ-CL[b] AS-ATV-PG[b] ATM-LM[b]	Oral - AS IV- AS IM- ATM	777 mg - AS 307 mg - ATM	Treatment of confirmed cases most of all as re-treatment (GW 3-12)	44	Abortion rate was 18.9% within the community range (12.3%). All infants were born externally and neurologically normal [60].
AS-ATV-PG	Not reported	840 mg - AS	Treatment of multidrug- resistant *P. falciparum* (GW not reported)	3	There were no congenital abnormalities or increased maternal adverse effects related to treatment [61].
ATM	IM	480 mg	Treatment after failure of chloroquine or quinine (GW 10)	1	There was no abortion, stillbirth or any congenital malformation [62].
ATM AS-PSD ATM-LM	IM – ATM Other oral	Not reported	Treatment after failure of uncomplicated cases (GW 6-12)	62	Most of the women enrolled received ATM injections and had normal babies followed until one year after birth [63].

AS: artesunate; ATM: artemether; PSD: pyrimethamine-sulfadoxine; MQ: mefloquine; CL: chloroquine ATV: atovaquone; PG: proguanil; LM: lumefantrine; GW-gestational weeks; LMP: last menstrual period; DHA: dihydroartemisinin; PQ: piperaquine; IV: intravenous. IM: intramuscular.

Table 3. Continued

Drug	Route	Total dose[a]	Exposure	Pregnant women	Effects observed
ATM-LM; ATM-LM-PSD	Oral[c]	480 mg[c]	Treatment of episodes of fever most of all unconfirmed by diagnostic tests (GW – LMP until 12)	156	The treatment with AL did not enhance perinatal mortality or impair infant neurodevelopment. The incidence of malformations was 6.9 % (mostly umbilical hernia) which was not higher than the incidence reported for the area. There was 4.5% of abortion after AL exposure which is not higher than spontaneous abortion rate data [64]. No effects on perinatal mortality or infants development until one year in a prospective cohort study [65].
Artemisia annua tea	Not reported	Not reported	Treatment of malaria episodes (GW not reported)	54	Two miscarriages were reported which was considered a low rate and probably not higher than the incidence in the population. All babies were normal [66].
ATM-LM	Not reported	Not reported	Inadvertent exposure for treatment of uncomplicated falciparum malaria (GW not reported)	96	There was a slightly higher rate of abortion, perinatal mortality, stillbirth and premature delivery after treatment (in all trimesters) that could not be distinguished from the effects of acute malaria itself. No adverse effects to fetuses or newborns [67].
AS[d]	Oral	930 mg[d]	Treatment of a single episode of malaria (GW <14 weeks)	64	24 miscarriages (31%) in the group exposed to AS which was not different from quinine or chloroquine treatment. No other adverse effect was related to treatment [68].
AS DHA-PQ AS-DHA-PQ	IV-AS Oral ACT	630 mg - DHA in ACT	Treatment of severe malaria (GW not reported)	18	There were 5 cases of miscarriage in women that received DHA-PQ (62.5% of 8 patients) compared with 2.6% (1/38) which received quinine [69].
ATM-LM ATM-LM-QUI	Oral	Not reported	Treatment of malaria, it was not mentioned if there were confirmed cases or the severity (GW 3-12)	172	There were 12.3% of miscarriage/stillbirth but the ACT was not associated with increased risk of any adverse pregnancy outcome. There were 0.6% of congenital anomalies which was not higher than global prevalence [70].

AS: artesunate; ATM: artemether; PSD: pyrimethamine-sulfadoxine; MQ: mefloquine; CL: chloroquine ATV: atovaquone; PG: proguanil; LM: lumefantrine; GW-gestational weeks; LMP: last menstrual period; DHA: dihydroartemisinin; PQ: piperaquine; IV: intravenous. IM: intramuscular.

Table 3. Continued

Any ACT	Not reported	Not reported	Inadvertent treatment of malaria confirmed or not (most of all GW 6-12)	299[e]	There was no increased risk of miscarriage in confirmed exposure (133 women). No fetal outcome was evaluated [71].
AS-MQ ATM-LM AS-CL AS DHA-PQ	Oral or parenteral	Not reported	Treatment of Falciparum malaria as a first choice or after quinine failure and ACT (GW 3-11)	312[f]	There was no increased risk of miscarriage, even when it was considered just the exposure on embryo-sensitive window. There was also no increased risk of any major congenital malformations in comparison to quinine [72].

The studies are presented in chronological order.

[a]The mean total dose was reported by the authors or corresponds to the amount of all dosage/day received per women, if it was described in mg/kg it was considered as a person of 70 kg.

[b]Artesunate or artemether were administrated alone or in combinations, different dose regimens were used and they were classified together as primary or re-treatment (43 and 57% of patients on first trimester, respectively).

[c]It was considered the dosage recommended for an adult at the label of Coartem® (Novartis), since the authors mentioned that its recommendations was followed.

[d]AS was administered as monotherapy to 21 women and the others were exposed to different ACTs, one with DHA and all other combinations were with AS. The authors mentioned that the women received a range from 12 to 16 mg/kg as total dose of artesunate according to the severity of malaria.

[e]In this number it is considered confirmed and unconfirmed exposed women.

[f]183 women received any artemisinin derivative alone (AS) or in different combinations to initially treat a falciparum malaria episode in the first trimester and 129 received an artemisinin derivative (mostly AS or AS-CL) in the first trimester after quinine failure. In this number can be included data from other previously published studies in the same area [60, 61, 68].

AS: artesunate; ATM: artemether; PSD: pyrimethamine-sulfadoxine; MQ: mefloquine; CL: chloroquine ATV: atovaquone; PG: proguanil; LM: lumefantrine; GW-gestational weeks; LMP: last menstrual period; DHA: dihydroartemisinin; PQ: piperaquine; IV: intravenous. IM: intramuscular.

5. Artemisinin derivatives mechanisms of embryotoxicity

The first insights about how these drugs cause toxic effects on embryo development around 10 years ago from *in vivo* and *in vitro* studies which remark pale yolk sac and reduced number of erythroblasts on rat embryos [8, 41, 42]. These effects were subsequently confirmed (Table 1). White et al. [42] demonstrated that artesunate induces embryonic erythroblasts death and that the depletion of these cells is not restored, which reduces oxygenation through fetal tissues causing its embryo toxic effects.

The critical period for embryo toxic effects of artemisinin derivatives was recognized as GD 10-14 in rats, and within this short time GD 10 was the most sensitive period for teratogenicity and GD 11 for embryolethality [44]. This sensitive period corresponds to timing when primitive erythroblasts are prominent on fetal circulation in rats produced by yolk sac islands [73]. It corroborates depletion of erythroblasts involvement with the embryo toxic effects observed. Indeed, it was demonstrated by *in vitro* studies that DHA causes defective and arrested cell division in embryonic erythroblasts followed by apoptosis [74].

Artemisinin derivatives' mode of antimalarial activity is not fully elucidated. It is proposed that these drugs exert their effects by multiple mechanisms, which is very common for herbal products [75]. It has been shown that these drugs need to be activated before their action and this generates ROS. The activation is supposed to be mediated by heme or ferrous iron generating carbon-centered radicals. Activated artemisinin derivatives may damage the parasites directly through oxidative stress and/or interfere with proteins or mitochondrial functions (reviewed by Pandey and Pandey-Rai [75]).

The heme, ferrous iron and oxidative stress involvement with their antimalarial effects may be correlated to the toxic effects to erythroblasts. Erythroblasts and reticulocytes are cells with high heme synthesis in which ferrous iron is required. Both of these cells are artemisinin toxicity targets in animals [76]. Indeed, it was demonstrated that higher concentrations of radiolabeled artesunate were present in tissues involved in hemoglobin synthesis or degradation, such as blood and liver in the fetus [76, 77].

Mitochondria are a target for artemisinin derivatives and may be involved in erythroblasts and reticulocytes toxicity due to their role on cell death [76]. An herbicide inhibitor of heme synthesis (S-53492) affects mitochondrial function and increases iron deposits on embryonic erythroblasts leading to a pattern of embryo effects similar to that observed for artemisinin derivatives [78, 79]. Since iron is toxic for erythroblasts and also plays a role on the activation of artemisinin derivatives, it is likely that mitochondrial damaged is involved in embryo toxic effects of these drugs [42].

Besides antimalarial actions, artemisinin compounds have emerged as an anticancer option. There are several mechanisms whereby they can act on cancer cells, such as inhibition of angiogenesis or cell differentiation and cell death [29]. The antiangiogenic effect of artemisinin was demonstrated by an *in vitro* assay using embryonic stem cells. This effect was attributed to oxidative stress, down-regulation of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) [80]. It is worth to remember that many anticancer drugs are teratogens and some teratogens became useful tools for anticancer therapy including thalidomide and arsenic [81].

Overall, embryo toxic mechanisms of artemisinin derivatives have probably multiple targets as it happens for their antimalarial [22] or anticancer effects [29]. The better understanding of these mechanisms of embryotoxicity will help to develop new strategies to obliterate this developmental toxicity.

6. Embryotoxicity differences from non-clinical and clinical data

The non-clinical information that circulating primitive erythroblasts are the main target for embryotoxicity of artemisinin derivatives [44, 74] emphasizes the importance to consider species differences in the erythropoiesis during embryo-fetal development.

In mammals primitive erythroblasts are formed in the blood islands of the yolk sac and are the first red blood cell produced by the embryo. The progenitors of these cells in rodents are present in the yolk sac for around 48 hours and primitive erythroblasts are released into circulation (around GD 8,5 in mice and GD 10 in rats) where they proliferate by cell division until the liver starts definitive erythropoiesis (around GD 13 in mice and GD 14.5 in rats) [74, 82, 83]. The sensitive period for developmental toxicity of artemisinin derivatives in rats coincides with the period when this susceptible embryonic cell population is circulating [44, 74].

In humans primitive erythroblasts are formed by the yolk sac between 3 to 6 weeks of gestation and are circulating in the embryo around 4 to 9 weeks of gestation (reviewed by [83]), this latter is the supposed sensitive period for toxicity to artemisinin derivatives [76]. Thus, if these sensitive cells are formed over a longer period of time, which means longer than the typical treatment period (3 to 7 days [3]), then damaged cells would be replaced by newly formed cells, and the consequences

might not be as dire [84]. Supporting this idea recent findings indicate that less mature forms of primitive erythroblasts (type I and pre-type I, such as the cells found in the yolk sac) are less sensitive to DHA, than circulating type II or III primitive erythroblasts [74]. This hypothesis, which were raised by Clark and co-workers [84], could explain why in non-human primates, treatment with greater than 12 mg/kg of artesunate for 12 days was necessary to induce embryolethality and teratogenicity [46]. Therefore, dose and time of exposure may be differently relevant for humans. However, it should be taken in account that during nine months of pregnancy a women can get sick in endemic areas more than once.

All developmental non-clinical studies with artemisinin derivatives until now have been conducted in non-infected animals. Meanwhile, almost all clinical studies have been conducted in malaria infected pregnant women or unconfirmed cases of women exposed without knowing their pregnancy. Due to obvious ethical issues they do not have non-treated infected groups as well as non-infected treated pregnant women as controls. There is scanty information about the exposure to these drugs during the first trimester of pregnancy because of WHO use restriction [3]. These are some of the difficulties to compare data from non-clinical and clinical studies and to define the risks for pregnant women and their fetuses.

Could the parasite changes on the host alter artemisinin derivatives toxicity? It was demonstrated less toxicity of artesunate in infected than in non-infected animals [85]. As reviewed by Clark [76] the reduction on reticulocytes caused by artemisinin derivatives was also lower in malaria patients. Nevertheless, there is no information about this reduction on embryo toxic effects but it is reasonable to think that it could happen [76].

Furthermore, experimental malaria infection alters amino acid transplacental transfer and drug transporters [86, 87]. This can alter kinetics of antimalarial drugs and needs to be considered on ACTs toxicity evaluation. The effects of pathophysiological changes on embryotoxicity of artemisinin derivatives have not been evaluated so far. These alterations underlying malaria during pregnancy could be one reason for the differences on data from developmental toxicity between non-clinical and clinical studies about developmental toxicity of artemisinin derivatives.

Some possible theories for the differences in sensitivity for embryo-fetal developmental toxic effects of artemisinin derivatives observed in non-clinical and clinical studies are summarized in figure 2. Briefly, if the human embryos are susceptible to the toxic effects of these drugs but the time of exposure is shorter than the period when the target cells (primitive erythroblasts) are being formed, it should be expected that these cells can be restored without too harmful effects for the fetus. Furthermore, the higher concentration of artemisinin derivatives in infected erythrocytes [28], and perhaps more activation and consequently bind to parasite proteins [22], could lead to less distribution of drug to the embryo. Taken together, the consequences for the fetus of pregnant infected women treated with artemisinin derivatives could be significantly different from the ones observed in experimental uninfected animals.

Finally, until now there is no evidence about embryo toxic effects of these drugs in humans. Large clinical trials and the appropriate evaluation of inadvertent exposure in the first trimester of pregnancy are highly necessary. This will bring concise information about the safety of artemisinin derivatives in the critical sensitive period for toxicity. Besides that, there are many difficulties to assess information in

pregnant women, thus, non-clinical studies with infected animals might help evaluate the effects of the infection and treatment together.

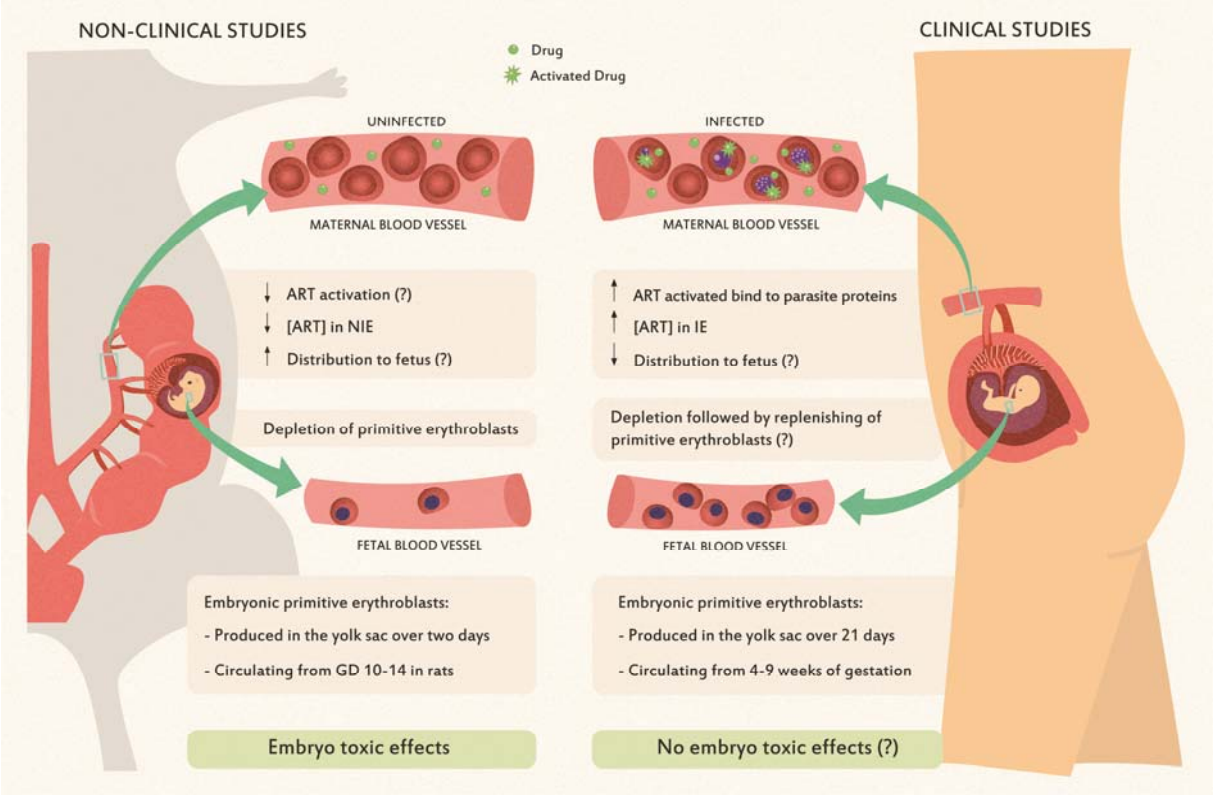

Figure 2. Differences between non-clinical and clinical studies about the safety of artemisinin derivatives use during pregnancy. The presence of malaria infection may imply in more activation of artemisinin derivatives (ART) by heme, which is provided mainly by parasite hemoglobin digestion, generating carbon-centered free radicals that are highly reactive molecules [20, 27]. These radicals can covalently bind to several parasite proteins alkylating them [22] and consequently being less available to pass to the embryo. In agreement with this hypothesis, it was shown that ART are more concentrated inside infected erythrocytes (IE) than in non-infected erythrocytes (NIE) [22, 28]. The mechanism of embryotoxicity of ART involves the depletion of circulating embryonic primitive erythroblasts [44, 74]. These cells are produced over approximately two days and are circulating from gestational day (GD) 10-14 in rats [44, 83]. These target cells are formed over a longer period of time, from 3-6 weeks of gestation, and they are circulating from 4 to 9 weeks of gestation in humans [76, 83]. Thus, considering a short treatment period (3 to 7 days [3]) in this estimated sensitive window for humans, the ART damaged cells may be replaced by newly formed primitive erythroblasts and the consequences to the fetus could be not as dire.

7. Conclusions and perspectives

There are a lot of gaps between non-clinical and clinical studies about developmental toxicity of artemisinin derivatives. Studies have several differences and it is very difficult to compare them and define the potential risks of the use of these drugs in the first trimester of pregnancy. Besides that, the risks of malaria in pregnancy seem to be higher than the ACTs adverse effects, and the effective treatment of the pregnant women is important to prevent malaria recrudescence. The mechanisms of embryotoxicity are not completely understood, but might not be so relevant for humans, considering the short time of treatment (3-7 days) compared with the longer period of target cell formation in the human embryo (~3 weeks). Apparently malaria infection might alter artemisinin derivatives activation and trap these drugs into the parasited erythrocyte, altering its distribution to embryo and consequently its embryo toxic effects. Further experimental studies using infected animals could be used to test this latter hypothesis. Large clinical trials and pharmacovigilance of pregnant women who receive these medications are essential. Thus, more information is needed to definitively allow artemisinin derivatives use during the whole pregnancy period.

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5.2 ARTIGO 2: *STANDARDIZATION OF A MALARIA MODEL DURING PREGNANCY IN SWISS MICE*

Aborda a padronização do modelo de malária e os efeitos da malária *per se* sobre o desenvolvimento fetal. Escrito nas normas do periódico *Reproductive toxicology*.

Standardization of a malaria model during pregnancy in Swiss mice

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Abstract

Malaria infection during human pregnancy causes low birth weight, intrauterine growth restriction, preterm delivery and maternal or fetal death. Upon these risks, infected pregnant women must be promptly treated. There are few antimalarial drugs considered safe to treat them, so it is highly necessary to develop new accessible treatment options for them. Such infection can cause modifications on host organs, which may change both the pharmacokinetics and toxicity profile of drugs. In the present study, we standardized a malaria model during pregnancy in Swiss mice which recapitulates the features of this infection in humans. A reduction in fetal body weight and skeletal growth retardation related to the infection were observed. Infected pregnant mice had preterm delivery, placental damage and alterations in multiple organs. This model will be helpful to better assess the effects of treatments during pregnancy, as well as the effects of malaria infection itself on the fetus development.

Keywords: malaria model, intrauterine growth restriction, skeletal teratology, placental histopathology.

1. Introduction

Malaria is a preventable disease that is still a worldwide public health concern. There are around 3 billion people at risk and 438,000 deaths caused by malaria each year [1]. Such infectious disease is caused by intracellular parasites of the *Plasmodium* genus, and it is transmitted by *Anopheles* mosquitoes. The majority of

cases occur in Africa where more than 300,000 children under five years died in 2015 due to malaria [1]. Pregnant women, infants and children are more susceptible to develop severe malaria [2, 3].

The consequences of malaria during pregnancy vary according to the *Plasmodium* species, the transmission intensity in the area and host factors, such as gravidity and immunity [4]. It has been described that in severe malaria, anemia and cerebral malaria are some maternal outcomes of the disease [2]. Miscarriage, stillbirth, preterm delivery, intrauterine growth retardation (IUGR), and low birth weight are some of the consequences to the fetus [2, 5]. *P. falciparum* is the species associated with the most severe forms of malaria in pregnancy, and this is attributed to the adherence ability of the infected erythrocytes (IEs) in the placental tissue [6]. However, *P. vivax* is also associated to poor pregnancy outcomes, and it is capable of inducing placental alterations [7-9]. The term placental malaria is used to characterize the malaria infections during pregnancy showing parasited erythrocytes on maternal intervillous spaces from the placental tissue, generally associated with inflammation [5].

Malaria is considered a clinical emergency, and pregnant women must be treated immediately. There are only few effective antimalarial drugs assessed to treat malaria during pregnancy [10]. The safety of the majority of antimalarial drugs in pregnancy is not very well characterized [11]. Nevertheless, as a result of malaria infection, severe adverse outcomes both for the mother and the child highly recommends the use of bed nets and intermittent preventive treatment whenever women get pregnant in areas with ongoing transmission [11]. Therefore, the risk to the fetus posed by treatments is a concern. Rather than the disease itself being harmful to the fetus, a better assessment is required in order to check the safety of

antimalarial drugs during pregnancy, trying to find best options with less adverse effects both for the mother and the fetus.

Clinical studies would be the best choice to assess antimalarial drugs already available and finding the risk-benefit ratio of each treatment for pregnant women [10]. However, there are a lot of limitations and difficulties to conduct studies on infectious diseases during pregnancy in humans, such as obvious ethical issues and problems related to the required data collection [12]. This way, the use of malaria experimental models is very interesting and helpful to assess the interactions between the host and parasite [13-15]. The mechanisms underlying placental malaria have been studied in experimental models, so they have been a useful tool to study the pathogenesis and immunological aspects involved in this illness [14]. The murine models of malaria using *P. berghei* were recapitulating some features of malaria in pregnancy caused by the *P. falciparum* in humans [14, 16-18]. These features include low birth weight, higher susceptibility to infection in pregnant animals, IUGR, decreased fetal viability and alterations on the placenta tissue [14, 16, 17].

Moreover, the emerging resistance of *P. falciparum* to the majority of antimalarial drugs available emphasizes the requirement for new antimalarial treatments. As the group at risk, pregnant women deserve more attention as to the development of new treatment options [10, 19]. Experimental malaria models have been largely used to assess the efficacy of antimalarial medications in non-pregnant animals [20] and more recently in pregnant animals [21]. All placental malaria models set until now use inbred animals [16-18, 21-24], however, besides some controversy [25], outbred animals are largely used in pharmacological and toxicological studies besides being more closer to the genetic variability found in humans [26, 27].

Based on such background, in this study, a malaria model was standardized during pregnancy in outbred Swiss mice using *P. berghei ANKA*. Furthermore, for the first time, the effects of malaria *in utero* exposure on skeletal development were assessed. Such model will be helpful in non-clinical studies for antimalarial treatments, providing information about their efficacy and toxicity during pregnancy and their developmental toxicity. It will also allow further assessment and a better understanding on the effects of malaria infection *per se* on the fetus development and long term adverse effects.

2. Materials and methods

2.1 *Animals*

Swiss Webster mice from the Federal University of Paraná were kept in standard water and food conditions (NUVILAB - CR1 from Nuvital, Brazil) *ad libitum*. 90 ± 10 days animals were acclimated for 10 days before the beginning of any procedure. In order to obtain pregnant females, two females paired with one male for 3 hours at the end of the dark cycle of light for mating. After that, the female was considered pregnant by the presence of vaginal plug and this determined the gestational day zero (GD 0). All the procedures with animals were previously approved by The Ethics Animal Experiment Committee of the Federal University of Parana under the number 648.

2.2 *Malaria model*

Mice erythrocytes infected by the *Plasmodium berghei* ANKA constitutively expressing green fluorescent protein (GFP) were prepared after successive

passages and kept at -80°C. Non-pregnant and pregnant females were infected by intraperitoneal injection in GD 0, 6 or 12 with 10^6 parasitized erythrocytes from frozen stocks. It previously demonstrated that pregnant females did not have successful pregnancies when infected before GD 12 [16]. To allow the assessment of the effects of the treatment before GD 12 two infection days before that were chosen for standardization. An uninfected group was formed by pregnant females receiving in GD 12 only intraperitoneal vehicle. The body weight was daily recorded after being infected. Parasitemia (the percentage of infected erythrocytes) was assessed by flow cytometry every 48 hours after infection, as described by Janse and Van Vianen [28]. For dams which did not survive until GD 18, a necropsy was performed to confirm pregnancy by visualization of the implantation sites.

2.3 *Cesarean section*

All pregnant females that survived until GD 18 were euthanized and the cesarean section was performed. The uterus was removed, weighted, and opened. Implantation, reabsorptions and fetuses were counted. The fetus viability was checked by touch reflection. Live fetuses and their placentas were separated, weighted, and kept for further analysis. Maternal kidneys, adrenals, spleen and liver were removed and weighted; for paired organs, the mean weight was used. The relative weights were calculated by multiplying the mean organ weight per 100 and dividing by the animal weight. Post-implantation loss was calculated by the amount of implantations less the amount of live fetuses multiplied per 100, then divided by the number of implantations.

2.4 *Assessment of skeletal teratology*

The fetuses were macroscopically assessed in order to check external abnormalities and then euthanized. The gender was determined by observing the anogenital distance. Half of each litter was randomly kept into a fixative (10% buffered formalin) for one month for further skeletal analysis. For this, a clarification and staining process was performed using Alizarin Red S following Staples and Schnell technique [29]. Skeletal examination was performed under a stereomicroscope and recorded following the Terminology of developmental abnormalities in common laboratory mammals (version 2) [30].

2.5 *Histopathologic and morphometric placental analysis*

Four placentas per litter (two from each gender of fetus) were fixed in 1.6% paraformaldehyde for 24 hours. This fixative was changed by 20% sucrose and in the following day, they were stored in 70% ethanol. Samples were processed to be inserted in paraffin, cleared and stained with hematoxylin-eosin. The histopathological analysis was performed under light microscope, and the alterations were scored from 0 to 3, according to their intensity. The amount of erythroblasts was counted in five fields of the labyrinth area under 400x magnification in one section for each placenta. Morphometric analysis of fetal blood vessel was performed using Image J (Image J 1.47v, National Institutes of Health, USA) in blind way. For this, photomicrographs (400x magnification 2070x1548 resolution) of three distinguished areas in the labyrinth region were taken from two non-consecutive sections from each placenta. The fetal lumen wall vessel area and the total fetal blood vessel areas of 18-24 cylindrical fetal blood vessels (characterized by an endothelial cell [31, 32]) were measured in these photos.

2.6 *Statistical Analysis*

Data were first analyzed for normal distribution and homogeneity of variances and then assessed according to the appropriate post-hoc tests. Differences were considered significant for $p \leq 0.05$. To assess teratology data it was calculated the mean percent of fetuses per litter with an external or skeletal abnormality, thus data were analyzed by Kruskal–Wallis test, followed by Dunn's test. Statistical tests are described in the graph or table, and the results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis and graphs were performed using Graphpad Prism® version 5.0.

3. Results and discussion

3.1 *Malaria model – Susceptibility to infection in pregnant females*

This study showed that malaria infection during pregnancy in Swiss mice reproduces some features of the placental malaria infection in humans, such as a higher susceptibility of pregnant females, reduced fetal body weight, intrauterine growth restriction, preterm delivery and histopathological changes in the placenta.

Pregnancy and time of infection during pregnancy influenced the susceptibility to the malaria; whenever the infection was in GD 0, the parasitemia was similar to the non-pregnant infected group (Figure 1). As the time of the infection was later in pregnancy (GD 6 or 12), the parasitemia increased and became even higher in the GD12-infected group with preterm delivery (Figure 1). Infection in GD 12 caused preterm delivery in 51% (11 females) of pregnant females in GD 17/GD 18, which did not happen in the uninfected group (Table 1). Preterm delivery was associated with parasitemia, anemia and increase in TNF-α and IL-10 levels in some clinical studies,

but the triggering mechanisms to such parturition remains unknown [33-36]. All infected pregnant females in GD 12 survived until GD 18, and have been euthanized. Although infected pregnant females in GD 0 and 6 presented lower parasitemias than the infected group in GD 12 (Figure 1b), these groups had no viable embryo or fetus, and their survival rates were not different from the non-pregnant infected females (data not shown). Such faster increases, both in parasitemia and mortality, in infected pregnant females during early pregnancy when compared with non-pregnant females have also been shown in others studies with different mice or *P. berghei* strains [16, 17, 23, 37, 38].

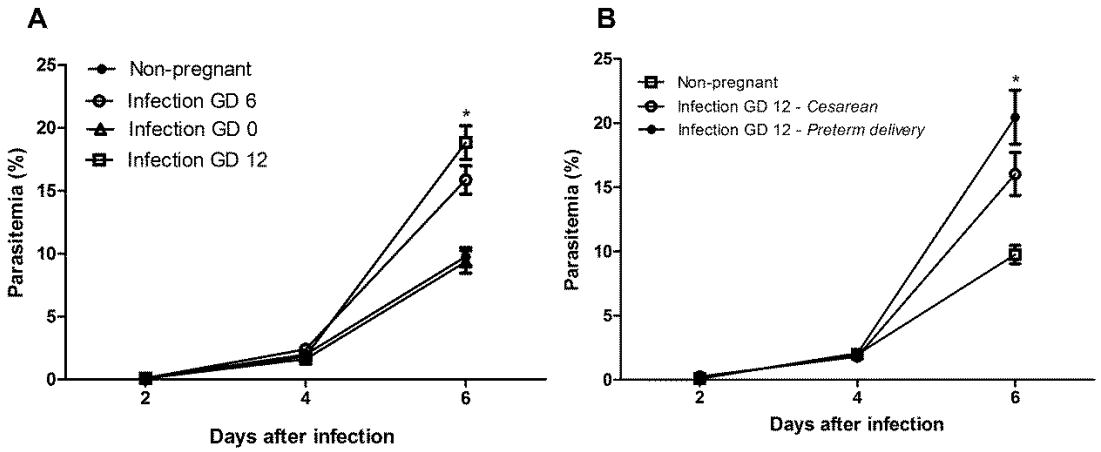

Figure 1. Parasitemia curves from pregnant and non-pregnant Swiss mice infected by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes. (A) Parasitemia of non-pregnant and pregnant females infected in the gestational days 0, 6 or 12 showing higher susceptibility of the infected pregnant females in GD 6 and 12. (B) Parasitemia curves of non-pregnant and pregnant females infected in the GD 12. Pregnant females who had preterm delivery presented higher parasitemias than those with normal gestational time till the cesarean section. Values are mean ± SEM, *$p<0.05$ when compared with the remaining groups (Two-way ANOVA following Bonferroni for A and B).

Table 1. Cesarean section (GD 18) data from Swiss mice infected by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes on GD 12.

	Uninfected	Infected
Pregnant females GD 0 (n)	23	21
Delivery in GD 17/18 (n)	0	11[#]
Cesarean in GD 18 (n)	23	10[#]
Implantations per litter (n)	14.4 ± 0.6	14.5 ± 0.5
Live Fetuses per litter[a] (n)	11.8 ± 0.7	12.8 ± 1.2
Reabsorptions per litter[a] (n)	2.6 ± 0.5	1.4 ± 0.5
Post-implantation losses per litter[a] (%)	17,2 ± 3.2	11.9 ± 5.9
Gravid uterus weight[a-b] (g)	20.3 ± 1.20	19.8 ± 1.7
Fetal body weight[a] (g)	1.28 ± 0.02	1.09 ± 0.03*
Placental weight[a] (g)	0.113 ± 0.005	0.114 ± 0.004
Fetal/placental weight ratio (g)[a]	11.8 ± 0.48	9.83 ±0.36*

Values are mean ± SEM.
[a]For these parameters, it was shown only data from pregnant females who had normal gestational time until cesarean section.
[b]Number of animals for gravid uterus weight was 21 due to a missing value.
*$p<0.05$ Unpaired *t* test.
[#]$p<0.05$ Fischer test.

3.2 *Effects on maternal body weight gain and on organs weight*

The body weight gain of infected pregnant females was significantly reduced after GD 16 (4 days of infection) when compared with the uninfected group (Table 2). Such effect corresponds to the time when parasitemia becomes more evident (Figure 1a-b). There was no significant difference between infected pregnant females from the cesarean and the uninfected group on the pregnancy weight gain after deducting the gravid uterus (Table 2). The reduction in maternal weight gain in the cesarean infected-group is probably due to intrauterine growth retardation observed through the reduced fetal body weight and skeletal growth restriction (Table 1 and 3).

There was an increase in maternal absolute and relative weights of spleen, liver, adrenals and kidneys in pregnant infected females from the cesarean group compared with the uninfected group (Table 2). Likewise, it was verified an increase in absolute weight of the spleen, liver and adrenals in the infected pregnant females from the preterm delivery group compared with the uninfected group (Table 2). When

they are considered together, the increased maternal organ weights in infected pregnant females observed here may be correlated to severe malaria infection. In humans, severe malaria often caused by the *P. falciparum* is a multiple organ disease which can result in anemia, acute kidney injury, pulmonary edema, hypoglycemia, lactic acidosis, liver dysfunctions, and cerebral malaria [11, 39-41]. The weight of the spleens was more than two times higher in the infected pregnant females than in the uninfected. Such finding is recognized in humans and in experimental infections and it is attributable to the immune response and removal of the parasitic erythrocyte functions of that organ [16, 42]. To our knowledge, there was no evidence on an increased weight of the adrenal from malaria experimental studies. An association between higher levels of cortisol and parasite load in primigravid women with placental malaria was reported [43]. Liver and kidney are important organs for metabolism and excretion, and so, alterations in these organs can change the pharmacokinetics or toxicokinetics of drugs. Some studies have demonstrated changes in the pharmacokinetics of antimalarial drugs in infected non-pregnant animals or in clinical trials [44-46], and pregnancy may alter the effectiveness and pharmacokinetics of antimalarial drugs [47-49]. Indeed, experimental malaria infections both by *P. berghei* (lethal) and *P. chabaudi* (non-lethal) parasites show induction of the CYP2a5 and down-regulation of the CYP1a and 2b in non-pregnant female mice [50]. This emphasizes the importance to address a pharmacokinetic profiling of antimalarial drugs in infected animals, and for this, the malaria model set here may be a valuable tool.

Table 2. Maternal body weight gain during pregnancy and maternal absolute/relative organ weight in GD 18 of infected individuals by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes.

	Uninfected	**Infected**	
		Cesarean	*Preterm delivery*
Pregnant females	23	10	11
Maternal body weight gain (Δg)			
GD 0-6	3.3 ± 0.22	3.4 ± 0.26	3.6 ± 0.48
GD 0-12	7.4 ± 0.29	7.4 ± 0.49	7.5 ± 0.45
GD 12 a 15	7.7 ± 0.47	8.4 ± 0.73	8.1 ± 0.51
GD 15-16	3.3 ± 0.24	3.5 ± 0.30	3.2 ± 0.28
GD 16-17	3.0 ± 0.23	1.5 ± 0.47*	1.9 ± 0.41*
GD 17-18	2.8 ± 0.20	0.15 ± 0.52[#]	[a]
GD 0-18	27.3 ± 1.01	24.3 ± 1.83	[a]
GD 0-18 (minus uterus weight)	7.1 ± 0.86	4.5 ± 0.68	[a]
Organ weights	20	10	11
Spleen absolute weight (g)	0.150 ± 0.006	0.354 ± 0.042*	0.298 ± 0.015*
Relative weight (%)	0.234 ± 0.012	0.580 ± 0.072*	[a]
Liver absolute weight (g)	3.2 ± 0.06	3.8 ± 0.19*	3.6 ± 0.10*
Relative weight (%)	4.9 ± 0.08	6.2% ± 0.20*	[a]
Adrenal absolute weight (g)	0.007 ± 0.0005	0.011 ± 0.0010*	0.010 ± 0.0006*
Relative weight (%)	0.011 ± 0.0007	0.017 ± 0.0015*	[a]
Kidney absolute weight (g)	0.227 ± 0.006	0.254 ± 0.009*	0.247 ± 0.008
Relative weight (%)	0.35 ± 0.009	0.41 ± 0.013*	[a]

Values are mean ± SEM.
*p<0.05 ANOVA - Bonferroni's Multiple Comparison Test or Unpaired *t* test (for relative organ weights) in comparison with uninfected.
[#]p<0.05 ANOVA - Bonferroni's Multiple Comparison Test in comparisons with all groups.
[a]Maternal body weight gain or relative organ weights were not calculated for infected preterm delivery group due to a significant reduction on maternal body weight before cesarean section.

3.3 *Effects on fetal intrauterine growth*

There were no alterations in the amount of implantations, live fetuses, reabsorptions, post-implantation losses or in gravid uterus weight related to malaria infection (Table 1). Fetal body weight was significantly reduced by the infection in pregnant females with normal time until the cesarean (Table 1). Low birth weight is a common consequence upon *in utero* exposure to malaria infection, especially by *P. falciparum* both in high and low transmission areas [2, 5, 51]. It is recognized that such effect on fetal weight can be due to fetal growth restriction or preterm delivery [33]. The reduced fetal body weight found in this study was also verified in other

malaria modelling using inbred mice [16, 17]. Furthermore, retardation on post-natal offspring development from infected females was verified after infection in the same gestational day and the inoculum size used here in the BALB/c mice [16].

It was not possible to verify if fetuses from infected pregnant females who had preterm delivery also presented low birth weight or other pregnancy outcomes because of cannibalism. But since their parasitemia was higher than in the infected pregnant females who had normal gestational time (Figure 1b), it is reasonable to think that it may infer in more severe adverse effects that should be assessed.

As mentioned before, IURG is one of the consequences of the placental malaria in humans [2, 5, 33, 36]. The present study confirms that malaria induces skeletal growth retardation (Table 3) displaying IURG. A consistent delayed ossification (incomplete ossification and unossified areas) was observed in fetuses from the infected group (Table 3). There was a significant increase of visible external abnormalities in the infected group with hematomas (Table 3). There was an increased incidence of sternebra 2 misshapen ossification sites and supernumerary cervical rib (short) in fetuses in the infected pregnant females (Table 3). These sternebra and rib alterations are commonly considered just variations of the bone ossification, and were considered as non-relevant to the malaria infection. There was a higher incidence of sternebra 6 misshapen ossification sites on fetuses as well from the uninfected group (Table 3). However, the upper incidence of unossified sternebra 6 observed in fetuses from the infected group reduced the probability to find an increasing misshapen ossification sites in such bone. Indeed, skeletal assessment showed no higher incidence of malformation related to the malaria infection (Table 3). It opens an opportunity to use this malaria model for further skeletal teratology studies with antimalarial drugs, allowing to distinguish the effects of the infection and

the treatment. Furthermore, it may be useful to analyze the effects of the treatment in the ameliorate intrauterine growth retardation. Therefore, the malaria model set here provides us an important tool for further investigations of both preterm delivery and intrauterine growth restriction mechanisms, which are until now not completely understood so far [2, 5, 36].

Table 3. Incidence of visible external and skeletal abnormalities in the offspring of Swiss mice infected by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes on GD 12.

	Uninfected	**Infected**
External abnormalities		
Fetuses/litters evaluated (N)	272/23	128/10
Litter mean%[a] (fetuses/litters) with abnormalities	20 (46/23)	50 (60/10)***
Eye open	13.3 (2/1)*	0
Subcutaneous Edema	1.16 (3/2)	1.48 (2/2)
Subcutaneous Hematoma	18.4 (41/15)	48.5 (60/10)***
Skeletal abnormalities		
Fetuses/litters evaluated (N)	139/23	61/9
Litter mean %[a] (fetuses/litters) with abnormalities:		
Skull bones		
Nasal, Unossified area[O]	1.97 (3/2)	17 (10/7)***
Frontal, Unossified area[O]	1.24 (2/2)	10.4 (6/5)**
Parietal, Unossified area[O]	19.7 (28/15)	44.3 (28/9)***
Interparietal, Unossified area[O]	48.2 (67/22)	65.5 (40/9)*
Supraoccipital, Unossified area[O]	42.3 (57/16)	65.2 (39/8)***
Supraoccipital, Misshapen ossification site[O]	68.8 (92/18)**	50.9 (30/6)
Supraoccipital, Unossified[O]	0.72 (1/1)	4.63 (3/2)
Forelimb bones		
Metacarpus 5, Unossified area[O]	6.62 (9/6)**	0
Proximal forepaw phalanx, Unossified[O]	19.5 (23/9)	48.7 (29/8)***
Ribs		
Rib, Supernumerary cervical rib (short) [V]	24 (33/13)	42.5 (25/8)**
Sternebra		
Sternebra 2, Unossified area[O]	7.78 (11/4)	19.4 (11/5)*
Sternebra 3, Unossified area[O]	14.1 (18/8)	27.9 (16/6)*
Sternebra 4, Unossified area[O]	30.1 (39/15)	44.3 (26/8)*
Sternebra 5, Unossified area[O]	55.3 (72/21)	67.9 (41/9)
Sternebra 6, Unossified[O]	0	9.92 (6/5)***
Sternebra 6, Misshapen ossification site[V]	98,4 (136/23)	88.4 (54/9)***
Sternebra 2, Misshapen ossification site[V]	50,2 (70/16)	66.7 (40/7)*
Hindlimb bones		
Hindpaw, Totally unossified[O]	0	5.09 (3/3)*
Metatarsus 1, Unossified area[O]	65.9 (88/22)	50.5 (31/9)*
Metatarsus 2-5, Unossified area[O]	67 (89/22)	50.5 (31/9)*
Metatarsus 2-5, Unossified[O]	0	5.09 (3/3)*
Proximal hindpaw phalanx, Unossified area[O]	67.2 (98/21)***	37.7 (23/9)
Proximal hindpaw phalanx, Unossified[O]	24.8 (30/12)	58.1 (35/9)***
Calcaneus ossified[O]	5.98 (10/2)*	0

[a]Litter Mean % = Average percentage of affected fetuses per litter in each group.
[V]Variation. [O]Alterations in the ossification. *p<0.05; **p<0.01; ***P<0.001.

3.4 *Histopathologic and morphometric placental alterations*

A reduction of fetal body weight was observed with no alterations in placental weight in infected pregnant females from the cesarean group (Table 1). In accordance with these results, the fetus/placental weight ratio was reduced in infected pregnant females from the cesarean group when compared with the uninfected group (Table 1). Fetal/placental weight ratio is a marker of placental efficiency: a larger placenta proportional to the fetus weight may indicate impairment of placental nutrient transport to the fetus, and it has been associated with low birth weight, spontaneous preterm delivery and stillbirth [52-54]. Studies in areas with high and low malaria transmission demonstrate a reduction in such ratio on primagravidae [24, 55]. Placental insufficiency is hypothesized as one of the main factors leading to fetal restriction growth and preterm delivery, resulting in a reduced fetal weight in newborns from pregnant women with placental malaria [55]. The reduction of the fetal/placental weight ratio observed in the present study corroborates such hypothesis.

Placental malaria in humans is evidenced by parasite erythrocytes and malarial pigment deposits in maternal vascular spaces, and it is associated with excessive perivillous fibrinoid deposits, thickening of the trophoblastic basement membrane, syncytiotrophoblast necrosis, increased syncytial knotting and inflammatory cell infiltrates in intervillous spaces [55]. In this study, the histopathological findings recapitulate many of these placental alterations. Placentas from the infected group exhibited parasites in maternal vascular spaces and brown pigmented fragments with eosinophilic deposition in maternal vascular space areas considered as parasites and/or hemolyzed erythrocytes debris (Figure 2b-c). It is important to point out that these parameters were not present in the placentas from

the uninfected group (Figure 2a,e). There was a significant upper fibrinoid deposition in the maternal decidua region in the infected group when compared with the uninfected (Table 4 and Figure 3). The placental labyrinth area showed a significant qualitative increase in mononuclear (Figure 2d) and polymorphonuclear cell infiltrates (Table 4).

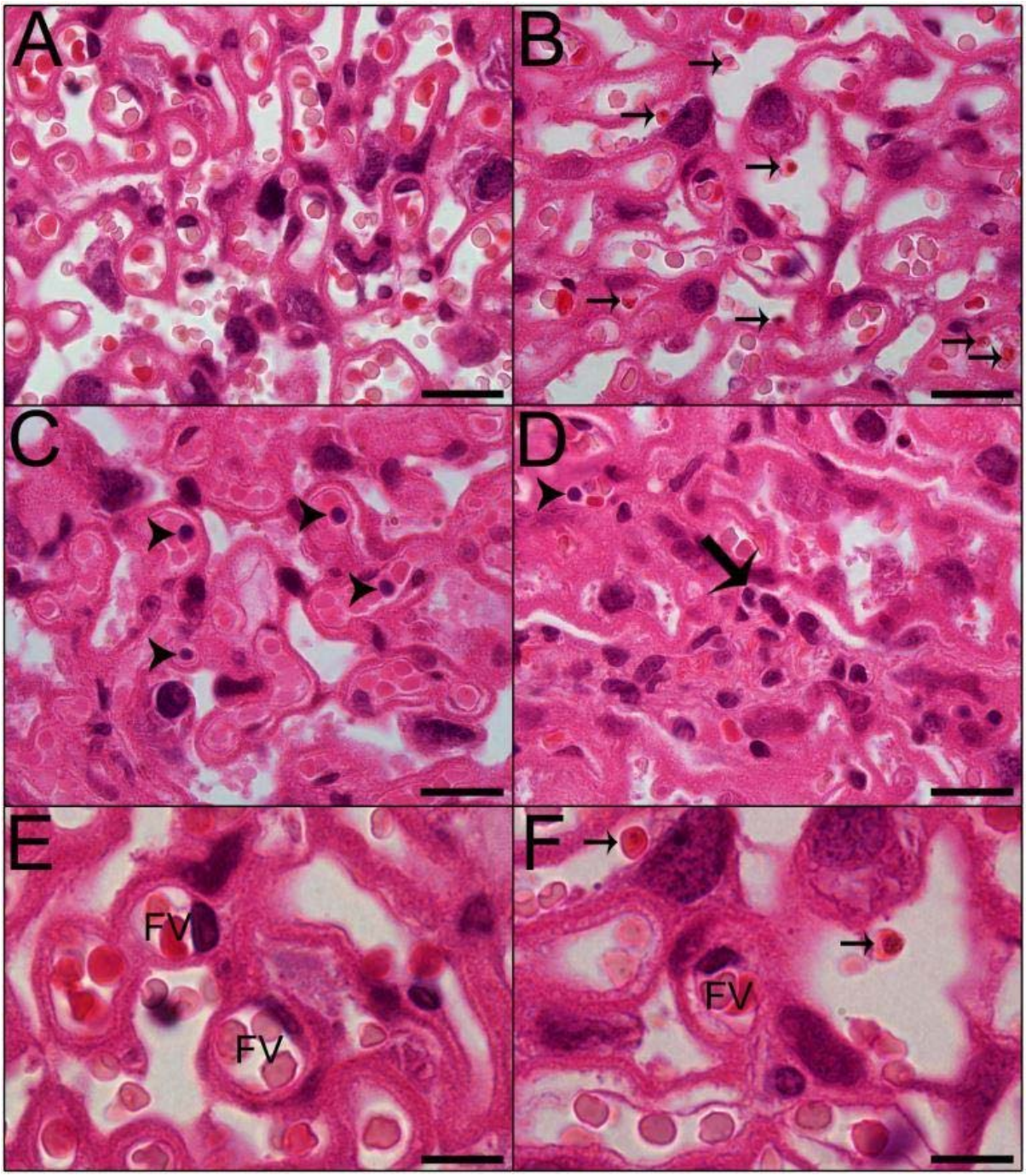

Figure 2. Parasites in maternal vascular spaces and increased fetal erythroblasts in placentas of pregnant mice exposed to the malaria infection. Representative histological sections from the uninfected placentas are shown in A and E. Parasite erythrocytes (small arrows in B and F), parasites and/or hemolyzed erythrocytes debris (eosinophilic deposition with brow pigments in the maternal vascular spaces observed in C), higher fetal erythroblasts (head arrows in C and D) and mononuclear cell infiltrates (pointed by the arrow in D) were observed in the placentas from infected pregnant mice. The higher thickness of fetal wall vessel in the infected group is observed in F. FV: fetal vessel. HE stains. Scale bar represents 20 μm in A-D and 40 μm in E-F.

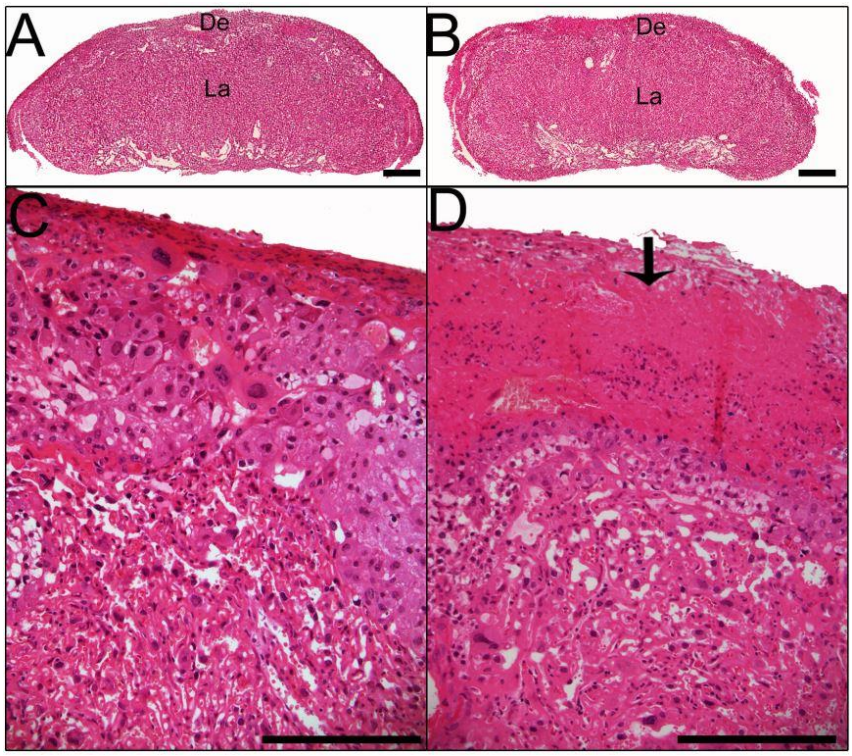

Figure 3. Higher fibrinoid deposition in placentas of the infected pregnant mice. It shows representative placental sections (GD 18) of the uninfected group (A and C) with low fibrinoid deposition on maternal decidua (eosinophilic area pointed by the arrow) and from infected group showing upper fibrinoid deposition (B and D). La = labyrinth area and De = decidua. HE stains. Scale bar represents 500 µm in A-B and 200 µm in C-D.

Table 4. Histopathologic and morphometric changes in placentas from mice in GD 18 after *in utero* exposure to malaria infection (GD 12).

	Uninfected	Infected	p value
Histopathologic analysis[a]			
Fibrinoid deposition on maternal decidua	1.0 ± 0.001	1.6 ± 0.107[#]	0.001
Polymorphonuclear cell infiltrates	0.031 ± 0.031	0.250 ± 0.091[#]	0.041
Mononuclear cell infiltrates	0.03 ± 0.03	0.08 ± 0.12[#]	0.002
Amount of erythroblasts	0.75 ± 0.13	14.8 ± 3.65[#]	0.0007
Morphometric analysis[a]			
Fetal wall vessel area (µm) per litter	116 ± 2.1	124 ± 3.7	0.065
per fetuses	116 ± 2.4	124 ± 3.2*	0.048
Fetal lumen vessel area (µm) per litter	36 ± 2.6	44 ± 3.2	0.078
per fetuses	36 ± 2.0	44 ± 3.1*	0.029
Total fetal vessel area (µm) per litter	152 ± 3.8	168 ± 5.7[#]	0.043
per fetuses	152 ± 3.3	168 ± 5.4[#]	0.022

Values are mean ± SEM. There were no significant differences related to the gender of fetuses in any of these analysis and data are shown together.

[a]For histopathologic parameters, there was no differences between statistical analysis per fetuses or per litter, thus data are shown only per litter. 4 placentas/litter and 8 litters from uninfected and 6 litters from infected group were analyzed for all parameters showed. It means that 32 placentas were of fetuses from the uninfected group and 24 placentas from the infected group.

*There was statistical difference ($p<0.05$) between the groups analyzed by the Unpaired *t* test.

[#]There was statistical difference ($p<0.05$) between the groups analyzed by the Mann Whitney test.

An increased amount of erythroblasts in fetal vessels associated to the malaria infection was verified here (Figure 2c and Table 4, thus corroborating the data found in literature [16, 37]. An increased amount of erythroblasts in placentas at term is considered a non-specific alteration, and it has been correlated to hypoxia, intrauterine growth restriction, fetal anemia and some congenital infections [56, 57]. The increasing erythroblast counting in placentas or in fetal circulation shows a good correlation [58]. Additionally, the elevated erythroblasts counting in the umbilical blood cord were correlated to high levels of IL-6, an inflammatory cytokine released through Toll-like receptor activation, in human fetuses with acute hypoxia [59]. A recent study showed higher IL-6 levels in the peripheral circulation of infected pregnant females mice in GD 19 with placental damages [60]. Such relation between the IL-6 level and the erythroblasts count, as well as its effects on fetal growth after *in utero* malaria infection needs to be better assessed.

Morphometric analysis of fetal blood vessels in this study indicates a syncytiotrofoblast layer compromised by malaria infection which is seen by the increased thickness of the fetal wall vessel (Figure 2f), lumen area and higher total fetal blood vessel area (Table 4). The vascular blood space area from the labyrinth region was reduced in malaria-infected placentas in experimental models attributable to the thickening in the syncytiotrofoblast layer [16, 17, 60]. The increased fetal lumen vessel area did not differ from those studies since the maternal blood vascular spaces area is considerably more significant in the total vascular labyrinth area of the placenta. The increased fetal lumen vessel area (Table 4) might be a fetal endothelial vessel response to the cytokines released by the inflammatory cells in the maternal vessel spaces that causes a vasodilation, which was never recorded before and needs to be assessed. Its alterations on thickening the syncytiotrofoblast layer came

to an agreement with recent findings on the reduction of transplacental amino acids transport possibly elicited by the intervillous inflammation in infected pregnant women with *P. falciparum* who had low birth weight babies [24]. Furthermore, malaria infection in pregnant female mice altered the metabolism system or transporters in the feto-placental and maternal tissues that may change both maternal and fetal disposition to antimalarial drugs [22].

4. Conclusion

In conclusion, this study presented a standardized malaria model during pregnancy in Swiss mice that resembles the features of the disease in humans. These characteristics include higher susceptibility of pregnant females, reduced fetal body weight, preterm delivery and placental injury due to infection. This model represents a valuable tool to advance the preclinical studies for drug development. New drugs or intervention strategies to treat malaria are extremely necessary concerning the emergence upon parasite resistance, and groups at risk as pregnant women and infants should be given more attention [1]. This model might improve fetal and maternal outcomes assessment, as well as upcoming studies providing pharmacokinetics information on antimalarial treatments. This experimental model is also helpful for a better understanding on the genetic differences of the host susceptibility to malaria infection as well as the effects of malaria itself on the fetus development.

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5.2.1 Material suplementar do artigo 2

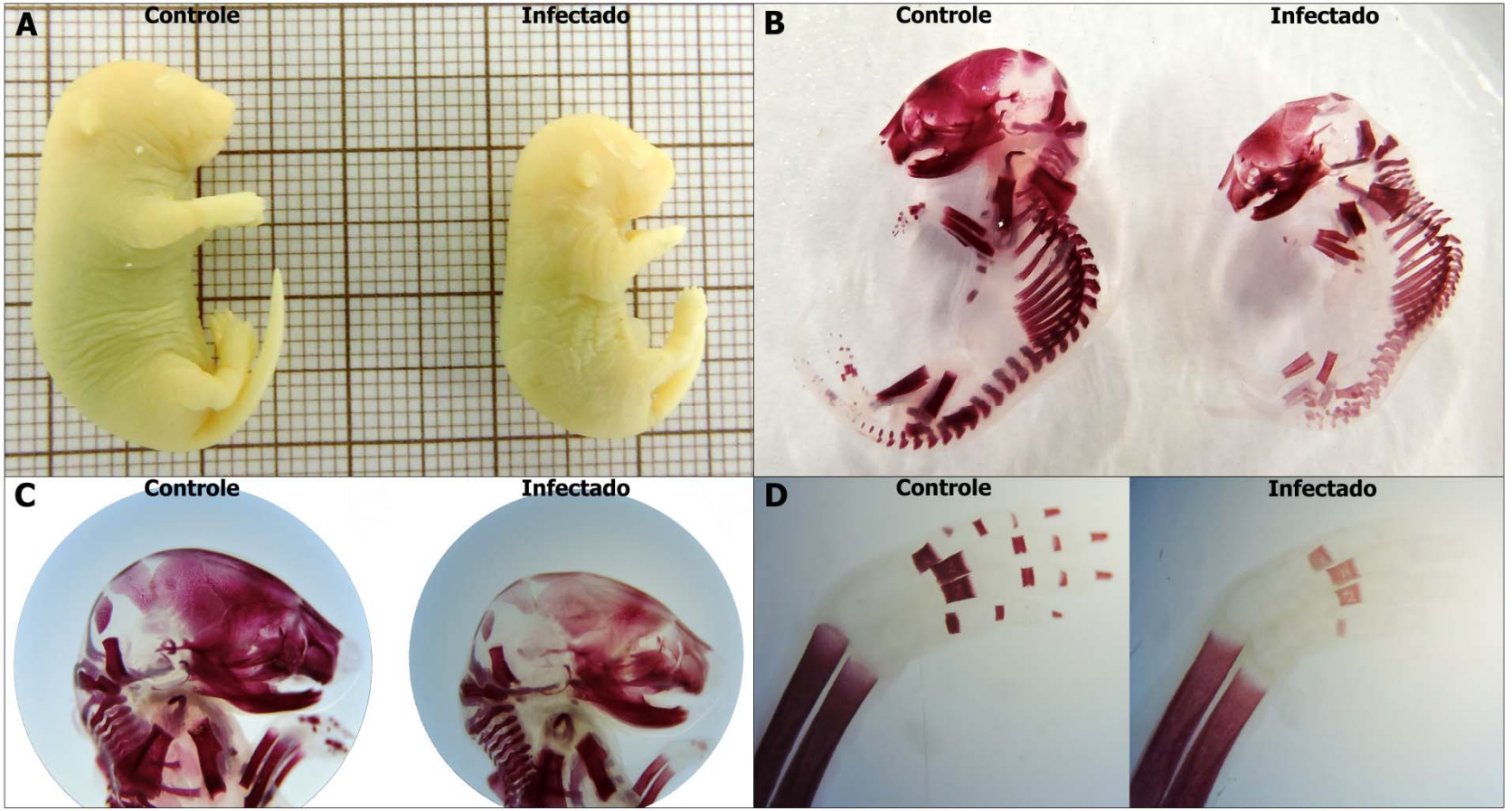

FIGURA 4: Efeitos da exposição *in utero* à infeção com 10^6 eritrócitos parasitados por *P. berghei* ANKA-GFP no 12° dia gestacional sobre o desenvolvimento fetal. Feto representativo do grupo não infectado (lado esquerdo de cada painel), externamente normal (A) e apresentado desenvolvimento ósseo adequado para a idade gestacional (18° dia) (B), esta ossificação normal é evidenciada em C para o crânio em D para os ossos dos membros anteriores. Feto do grupo infectado (no lado direito de cada painel) mostrando uma redução no tamanho (A), retardo no desenvolvimento ósseo (B), crânio pobremente ossificado (C) e ausência de falanges proximais e distais dos membros anteriores (D).

5.3 ARTIGO 3: EMBRYOTOXICITY OF ARTESUNATE IN AN EXPERIMENTAL MALARIA MODEL

Avalia o tratamento com o artesunato no modelo de malária previamente padronizado. Escrito nas normas do periódico *Reproductive toxicology*.

Embryotoxicity of artesunate in an experimental malaria model

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Abstract

Malaria during pregnancy is a very damaging disease to mother and fetus, requiring immediate treatment when diagnosed. Artemisinin derivatives, currently the most important antimalarial drugs, have their use restricted during the first trimester of pregnancy due to embryotoxicity observed in laboratory animals. In this work, we evaluated the embryotoxicity of artesunate, an important artemisinin derivative, in a malaria model, trying to minimize the differences in potential toxicity of infected and uninfected animals. As expected, artesunate reduces the maternal negative effects caused by the infection. Furthermore, the embryolethality caused by artesunate highest dose was reduced when it was administered to infected pregnant mice. These findings indicate that the embryotoxicity of antimalarial drugs can be changed by the presence of infection and the safety of artemisinin derivatives needs to be better evaluated in experimental malaria models.

Keywords: artesunate, artemisinin derivative, embryotoxicity, embryolethality, malaria model.

1. Introduction

Malaria is still a significant cause of morbidity and mortality in areas in which it is endemic. This disease is responsible for about 400,000 deaths per year, most of them in children from sub-Saharan Africa [1]. Miscarriage, anemia, cerebral malaria, preterm delivery, low birth weight and maternal or fetal death are some of the consequences of malaria in pregnancy [2, 3].

As a result of these risks pregnant women with malaria must be treated without delay with the best treatment available. The World Health Organization (WHO) recommends the use of quinine plus clindamycin to treat uncomplicated malaria caused by *P. falciparum* during the first trimester of pregnancy. Artemisinin-based combination therapies (ACTs) are the first choice treatment in the following trimesters. Furthermore the immediate treatment with artesunate followed by ACTs is approved to treat cases of severe malaria throughout gestation [4].

The restricted use of artemisinin derivatives in the first trimester of pregnancy relies on their possible embryo toxic effects. These drugs are recognized as developmental toxicants on animals causing embryolethality and teratogenicity on a range of doses. Several studies have demonstrated these effects on different animal species including monkeys [5-10]. The mechanisms of embryotoxicity involve the depletion of primitive erythroblasts which are circulating from gestational weeks 4 to 9 in humans [4, 9, 11, 12].

Besides that, until now there is no evidence that artemisinin derivatives cause any adverse effect to human fetus or neonate. Nevertheless, there are just a few clinical studies with artemisinin derivatives in the first trimester of pregnancy and they

have together a small number of pregnant women evaluated to detect statistically significant increases on negative fetal outcomes [4].

One of the differences between clinical and non-clinical studies is the presence of infection. Most of clinical studies were conducted with pregnant women treated for malaria (even if not confirmed) while the experimental studies were conducted in uninfected animals [13]. The pathogenesis of malaria in pregnancy has been extensively studied in recent years using experimental models. The murine models using *P. berghei* were resemble many features of malaria in pregnancy caused by *P. falciparum* in humans, such as placental alterations, a reduction on fetal weight and higher susceptibility to infection in pregnant animals [14-19]. Experimental malaria models also showed that metabolizing enzymes and placental transporters can be altered by the infection [20, 21]. Then, it is reasonable to think that the infection can alter the toxicokinetics of antimalarial drugs.

Indeed, the exact antimalarial mechanism of action of artemisinin and its derivatives remains not defined until now but may be relevantly altered in infected patients. There are many questions about their mode of activation and molecular targets. Recently Wang et al. [22] demonstrated that heme is more important than ferrous iron on the artemisinin activation and that the activated drug binds to several parasite proteins important for their survival. The source of heme is supposed to be provided by parasites biosynthesis during the initial parasite stages and mainly by hemoglobin digestion on latter stages. Thus, drug activation and its pharmacokinetics may be different between healthy and infected people and could account for some selective toxicity of these drugs to parasites [22]. This is supported by literature data in which higher concentrations of artemisinin derivatives were found in plasma from malaria infected patients rather than in healthy subjects [13]

Considering these literature data Clark [13] noted that the toxicity of artemisinin derivatives can be altered by malaria infection, leading to a reduction on their distribution to target tissues. In this context, we evaluated the developmental toxicity of artesunate, a widely used artemisinin derivative, using a malaria model.

2. Materials and methods

2.1 *Animals*

All following procedures were previously approved by the Ethics Animal Experiment Committee of Federal University of Paraná (number 648). Male and female Swiss Webster mice (90 ± 10 days) were obtained from Federal University of Paraná and kept in standardized conditions with water and regular food (NUVILAB - CR1 from Nuvital, Brazil). The animals were placed together in proportion of one male for two females during three hours in the end of the dark cycle for matting. Afterward females with vaginal plug were considered pregnant and this was considered as gestational day zero (GD 0).

2.2 *Experimental* m*alaria model during pregnancy*

Pregnant mice were infected in GD 6 by intraperitoneal injection of 10^6 parasited erythrocytes with *P. berghei* ANKA constitutively expressing green fluorescent protein (GFP) from frozen stocks. This infection day was determined after we confirmed that pregnant infected females survived until GD 12 in a standardization of a malaria model (unpublished data). Maternal body weight was verified every day after infection. Parasitemia was evaluated by flow cytometry every 48 hours after infection as previously described [23].

2.3 *Treatment groups*

Pregnant mice were divided in two major groups (infected and uninfected) for comparison. Each major group was subdivided in females that received vehicle (carboxymethyl cellulose 0.5% in distillated water) or artesunate (Sigma-Aldrich) in the doses 7.5; 15 or 30 mg/kg. The highest dose of artesunate (30 mg/kg) was estimated by allometry based on the human therapeutic dose (4 mg/kg/day) extrapolated to mice [24]. Because of experimental studies showing increased resorption in doses higher than 20 mg/kg in mice, the other lower doses were determined [7]. The administrations were done in GD 10 by oral route in a volume of 10 mL/kg. GD 10 was considered the most sensitive day for teratogenic effects of this drug in rats [11]. All artesunate solutions were freshly prepared.

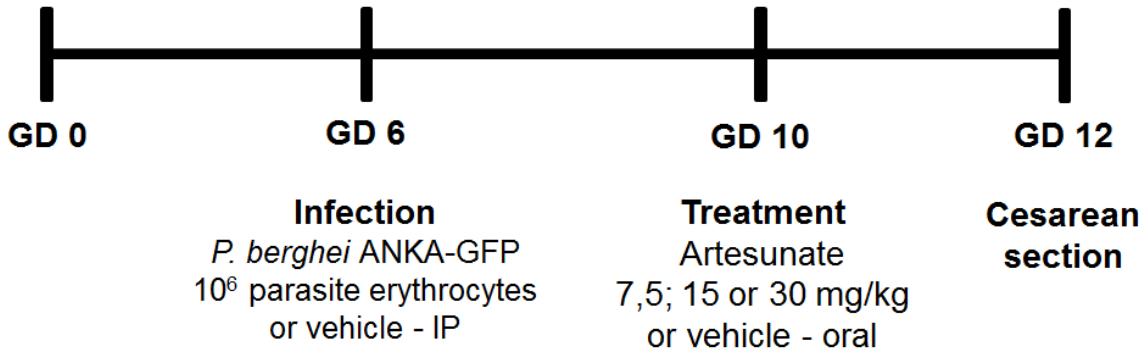

Figure 1. Experimental design.

2.4 *Cesarean section*

All pregnant females were euthanized in GD 12. The uterus, spleen, liver, kidneys and adrenals were removed and weighted. Each uterus was carefully opened and the yolk sacs with embryos were removed under a stereomicroscope. The vascularization color of each yolk sac was immediately checked. Resorptions

were recorded. After that, each embryo was removed from their yolk sac and their viability was confirmed by heart beating. Two randomly viable embryos per litter were separated for embryo histopathology analysis. Post-implantation losses were calculated as the amount of implantations less the amount of live embryos multiplied per 100, then divided per number of implantations. The relative weights were calculated by multiplying the mean of organ weight per 100 and dividing by the animal weight.

2.5 *Embryo histopathologic evaluation*

The selected embryos were processed as described by Boareto et. al. [6]. Briefly the embryos were fixed in Methacarn (methanol 60%, chloroform 30% and acetic acid 10%) for 24 hours at 4°C. They were dehydrated, diaphanized and embedded in paraffin. For the histopathological analysis one live embryo from five or six pregnant females per group were completely sectioned in sagittal segments of 5 µm thickness. The sections were stained with hematoxylin and eosin. The slices from the control groups (uninfected and infected) and from the groups infected and uninfected followed by artesunate 30 mg/kg were analyzed under light microscope. It was determined the histopathological indexes as designated before with slight modifications [6, 25]. For each histopathological alteration observed, scores were given from 0 to 4 (according to the severity) and they were grouped within the following patterns: red blood cells (RBC), heart, liver, central nervous system (CNS), peripheral nervous system (PNS) or alterations in any other tissue/organ than these cited patterns.

The histopathological index for each alteration was calculated by the sum of the scores multiplying per 10 and per the importance factor. Since it is not defined

the importance of each alteration on embryo survival we considered them all equally important (using the factor of three) except for architectural tissue organization which was rarely found and very slightly (factor of one). The histopathological index in the pattern was given by the sum of each alteration index divided per number of alterations observed on the pattern. The histopathological index per embryo was obtained by the sum of all pattern indexes. Thus, the results were expressed as a prevalence of histopathological alterations in each pattern per embryo, or in the whole embryo per group. Photomicrographs were taken with a Leica EC 3 camera attached to a Leica DM 2500 light microscope and the software Leica Application Suite – LAS EZ.

2.6 *Statistical analysis*

Data were first analyzed for normal distribution and homogeneity of variances and then assessed according to the appropriate post-hoc tests. When data had normal distribution, they were analyzed by ANOVA (variance analysis) followed by Bonferroni post-hoc test. Data with abnormal distribution were analyzed by Kruskal-Wallis followed by Dunn's test. To compare the differences between infection and treatment the respective groups were analyzed by Two-way ANOVA followed by Bonferroni. The histopathological indexes were analyzed by Kruskal-Wallis followed by Dunn's test. The significance level considered was 5% ($p < 0.05$). Statistical tests are described in each graph. Data are expressed as mean ± standard error of mean (SEM). Statistical analysis and graphs were performed using Graphpad Prism® version 5.0.

3. Results

3.1 *Efficacy of artesunate in a malaria model during pregnancy*

One pregnant mouse died before cesarean (GD 12) due to infection. Artesunate was effective and the parasitemias of treated groups in GD 12 (6 days after infection) were lower than parasitemias of infected group (vehicle) in a dose-response manner (Figure 2a). Artesunate treatment in all doses prevented the increase of kidneys weight observed in the infected group (Figure 2c), but this protective effect did not occur for liver weight (Figure 2d). Interestingly, the expected increase of spleen weight due to infection was even more pronounced in pregnant mice treated with artesunate 15 or 30 mg/kg (Figure 2b). There was no difference on adrenals weight among the groups (data not shown).

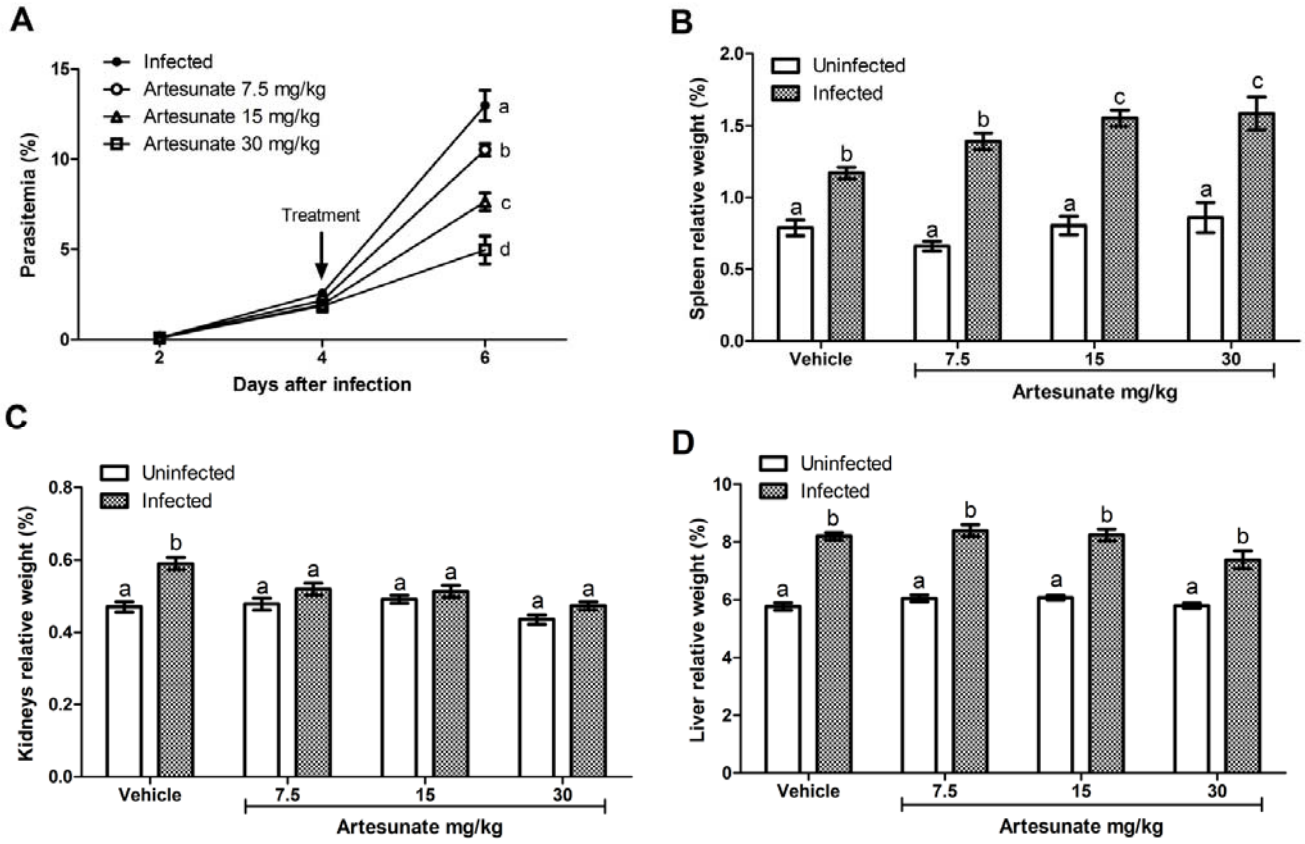

Figure 2. Efficacy of artesunate in a malaria model during pregnancy. Artesunate treatment (GD 10; 7.5, 15 or 30 mg/kg) prevented the increase in parasitemia in pregnant females infected by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes in GD 6 (A). The other panels show the effects of artesunate treatment in the relative weight of spleen (B), kidneys (C) and liver (D) in infected and uninfected pregnant females. Data are expressed as mean ± SEM. Each group had 9-11 pregnant females. Different letters represent statistically different groups ($p<0.05$). One-way ANOVA followed by Bonferroni were used to analyze differences among infected groups and uninfected vehicle; or among uninfected groups (vehicle and artesunate treatments). Two-way ANOVA followed by Bonferroni were used to compare differences between infected and uninfected respective treatment group.

3.2 *Effects of artesunate treatment on pregnancy outcomes*

There was no difference on maternal body weight gain due to infection or treatment from before GD 10 (Table 1). The artesunate treatment did not affect the maternal body weight gain in uninfected pregnant mice (Table 1). The infection reduced significantly the maternal body weight gain from GD 10 to 12 and this was prevented by artesunate treatment from GD 11-12 (Table 1).

There was no difference among the groups on number of implantations (Table 1), resorptions or on gravid uterus weight (data not shown). There was a reduction on the percentage of live embryos, caused by either the infection or artesunate (in uninfected animals) at the highest dose (Table 1). This reduction on percentage of live embryos caused by the infection was prevented by artesunate treatment in all doses (Table 1). Although, there was no difference on the percentage of live embryos between uninfected or infected groups which received artesunate highest dose, the mean was slightly higher in artesunate infected group (59% and 84% respectively, Table 1).

The infection increased the post-implantation losses, which were reduced by all doses of artesunate. The artesunate highest dose isolated also increased post-implantation loss and there was no statistical difference due to infection on the groups that received this dose (Table 1). Overall, there was an increase on the percentage of total litter loss (which means litters with dead developed embryos) caused by the infection or by the treatment with artesunate 30 mg/kg, this outcome did not happen in the infected group treated with the same dose (Table 1). Embryos from pregnant mice treated with artesunate highest dose presented remarkable pale yolk sacs independent of the infection (Table 1 and Figure 3).

Table 1. Maternal and pregnancy data (C-section GD 12) of pregnant females treated with artesunate (GD 10) and uninfected or infected by *P. berghei* ANKA-GFP 10^6 parasite erythrocytes (GD 6).

Treatment	Uninfected				Infected			
	Vehicle	Artesunate (mg/kg)			Vehicle	Artesunate (mg/kg)		
		7.5	15	30		7.5	15	30
Pregnant females	10	10	10	11	9	10	10	10
Maternal body weight gain (Δg)								
GD 0-6	2.8 ± 0,47	2.5 ± 0.28	3.4 ± 0.27	2.8 ± 0.49	3.0 ± 0.27	3.8 ± 0.32	4.3 ± 0.45	2.9 ± 0.44
GD 6-10	3.0 ± 0.38	3.6 ± 0.28	2.9 ± 0.40	3.6 ± 0.41	2.6 ± 0.45	3.3 ± 0.28	2.2 ± 0.27	3.0 ± 0.38
GD 10-11	1.40 ± 0.30	1.05 ± 0.27	1.50 ± 0.25	1.32 ± 0.18	0.35 ± 0.18[a]	0.75 ± 0.24	0.85 ± 0.24	1.25 ± 0.20
GD 11-12	1.50 ± 0.20	2.15 ± 0.30	1.45 ± 0.23	2.05 ± 0.30	-1.70 ± 0.21[a]	0.50 ± 0.24[b]	0.95 ± 0.35[b]	1.30 ± 0.20[b]
Mean litter values								
Implantations (n)	13.1 ± 0.55	13.1 ± 0.67	13.7 ± 0.91	14.0 ± 0.54	13.6 ± 0.81	13.9 ± 0.95	13.5 ± 0.60	11.4 ± 1.00
Live Fetuses[a] (n)	12.1 ± 0.67	11.2 ± 0.81	10.1 ± 1.09	6.6 ± 1.67[a]	3.6 ± 1.38 [a]	9.6 ± 1.28[b]	10.0 ± 1.31[b]	8.1 ± 1.02
Live Fetuses (%)	100 ± 0	99.3 ± 0.71	98.6 ± 1.43	58.8 ± 14.28[c]	27.7 ± 11,06[c]	73.2 ± 7.70[c]	85.3 ± 9.97[d]	84.2 ± 7.42[d]
Post-implantation losses per litter (%)	7.75 ± 3.09	14.3 ± 4.68	26.8 ± 5.90	52.8 ± 12.30[a]	76.7 ± 8.68[a]	31.2 ± 8.53[b]	24.0 ± 9.94[b]	29.4 ± 6.13[b]
Total litter loss % (n/litter)	0 (0/10)	0 (0/10)	0 (0/10)	36.4 (4/11)[c]	44.4 (4/9)[c]	0 (0/10)[d]	10 (1/10)	0 (0/10)[d/e]
Pale yolk sac (%)	0	0	3.3 ± 2.54	100[c]	2.5 ± 2.50	0 ± 0	13.7 ± 10.93	98 ± 2[c/d]

Data are expressed as mean ± SEM.
[a]p<0.05 compared with vehicle uninfected group (One-way ANOVA followed by Bonferroni). [b]p<0.05 compared with vehicle infected group (One-way ANOVA followed by Bonferroni). [c]p<0.05 compared with vehicle uninfected group (Kruskal-Wallis followed by Dunn's test). [d]p<0.05 compared with vehicle infected group (Kruskal-Wallis followed by Dunn's test). [e]p<0.05 compared with uninfected respective dose group (Two-way ANOVA followed by Bonferroni).

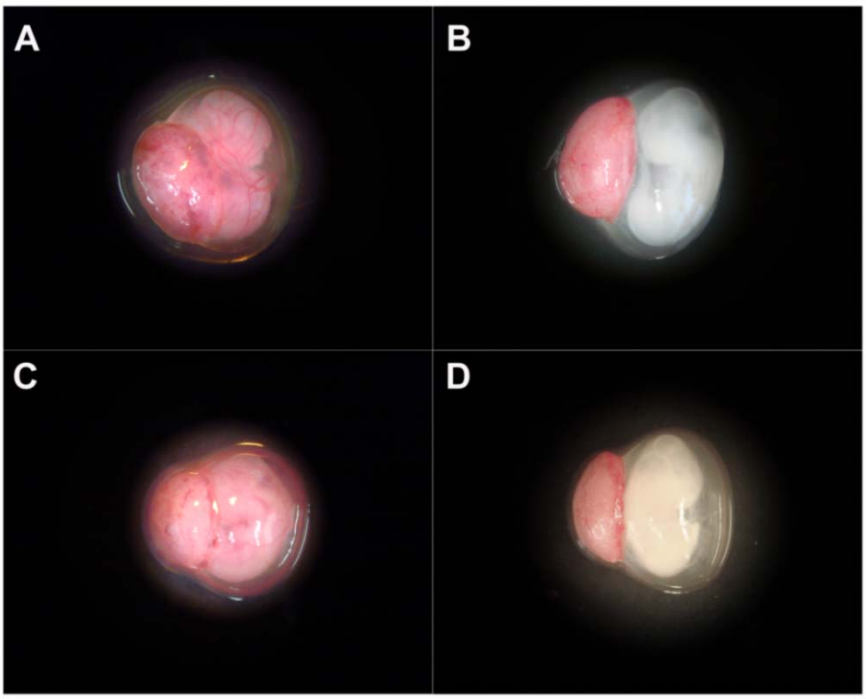

Figure 3. Remarkable pale yolk sac in embryos from GD 12 after administration of artesunate in GD 10 to uninfected and infected mice (*P. berghei* ANKA-GFP 10^6 parasite erythrocytes in GD 6). Representative embryos on their yolk sac showing normal colored yolk sac in embryos from uninfected (A) and infected (C) groups untreated on the left side. Embryos from pregnant uninfected or infected females treated with artesunate 30 mg/kg showing a marked pale yolk sac on the right side, B and D respectively.

3.3 *Effects of infection and artesunate 30 mg/kg on embryo histopathologic analysis*

All the control embryos presented preserved structures and moderate to high amount of RBC, with just small alterations on embryonic tissues in not as higher intensity or severity as in other groups (Figure 4; Figure 5a,c,e and Figure 6a,c).

The embryos exposed to artesunate treatment itself showed a significantly higher histopathological index for the patterns of RBC and liver (Figure 4). There were scarce erythroblasts circulating (the majority of vessels were empty, Figure 5f) and in it was seen apoptosis in these cells when they were in the liver. There was a reduction on liver size (Figure 5c-d).

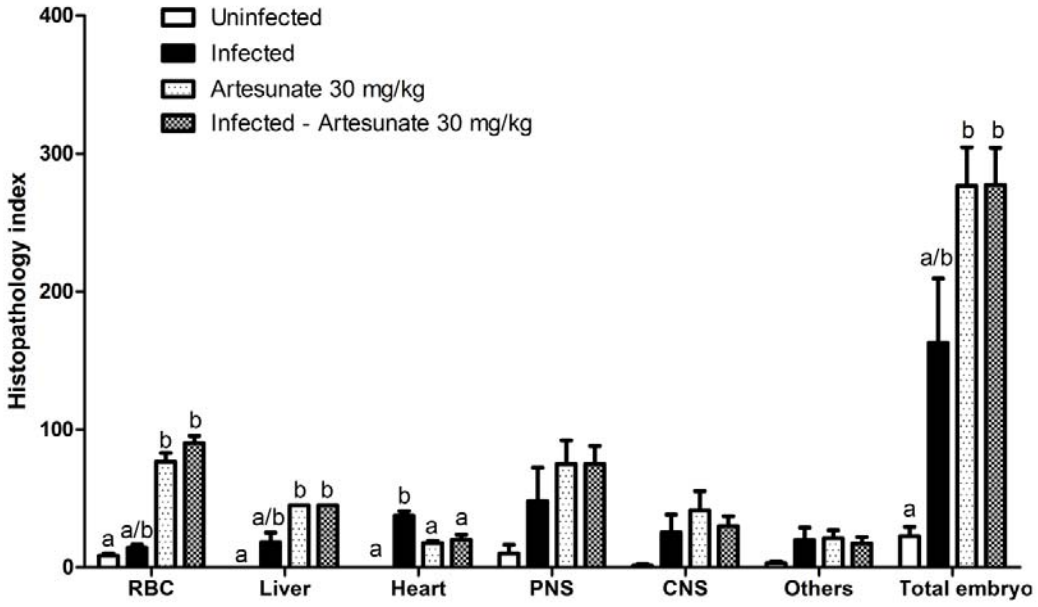

Figure 4. Histopathological indexes from embryos in GD 12 after *in utero* infection with *P. berghei* ANKA-GFP 10^6 parasite erythrocytes, treatment with artesunate or both. The indexes are shown for each analyzed pattern: red blood cells (RBC), liver, heart, peripheral nervous system (PNS), central nervous system (CNS), all other alterations observed per embryo (Others) and the total index per embryo (Total embryo). Different letters mean statistically different groups (Kruskal-Wallis followed by Dunn's test). There was no difference among the groups for the patterns without any letter. Data are expressed as mean ± SEM.

The effects of the infection itself on histopathological index by pattern was statistically higher for the pattern of alterations in the heart (Figure 4), which are represented mainly by apoptosis and a reduction on thickness of heart walls (Figure 5b). Although, there was no statistical difference, higher means were observed in histopathological indexes for this group in comparison with the uninfected group (vehicle) for these following patterns of alterations: CNS (25.5 ± 12.7 and 1.25 ± 1.25 respectively), PNS (48 ± 24.4 and 10 ± 6.3), liver (18 ± 7.4 and 0) and others (19.7 ± 9.2 and 3.1 ± 1.1). This higher intensity of alterations can be reflected by an increase in total embryo index (163 ± 47 for the infected group and 23 ± 6.8 for the uninfected-vehicle), even though it was not statistically different.

The histopathological indexes for all patterns were similar between infected and uninfected groups that received artesunate (Figure 4). It was observed the same

alterations on heart, liver or other alterations patterns (Figure 4b). There are some representative alterations shown in Figures 5d,f and Figures 6b,d.

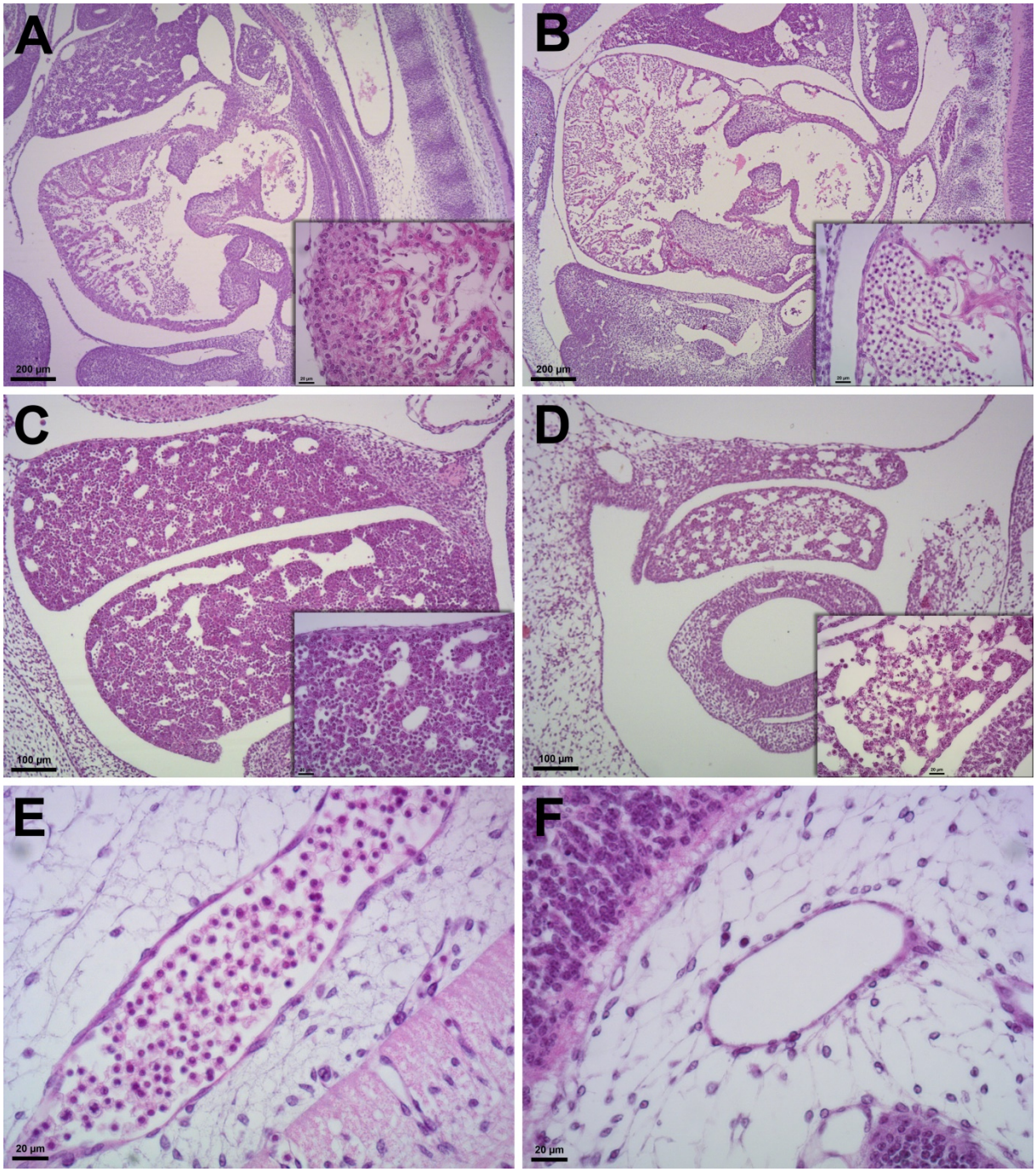

Figure 5. Representative alterations on heart, liver and red blood cells (RBC) in sagittal sections from mice embryos in GD 12 after *in utero* infection with *P. berghei* ANKA-GFP 10^6 parasite erythrocytes and treatment with artesunate. Control embryo showed normal heart (A) and liver (C). Embryo from infected group showed normal amount of RBC (E), but a reduction on thickness of heart walls (B) and apoptosis in heart tissue (insert B). Embryo from the group in which pregnant mice were infected and treated with artesunate 30 mg/kg showed a reduction in the size of liver (D) and in the amount of RBC (F); and apoptosis in RBC present in liver (insert C).

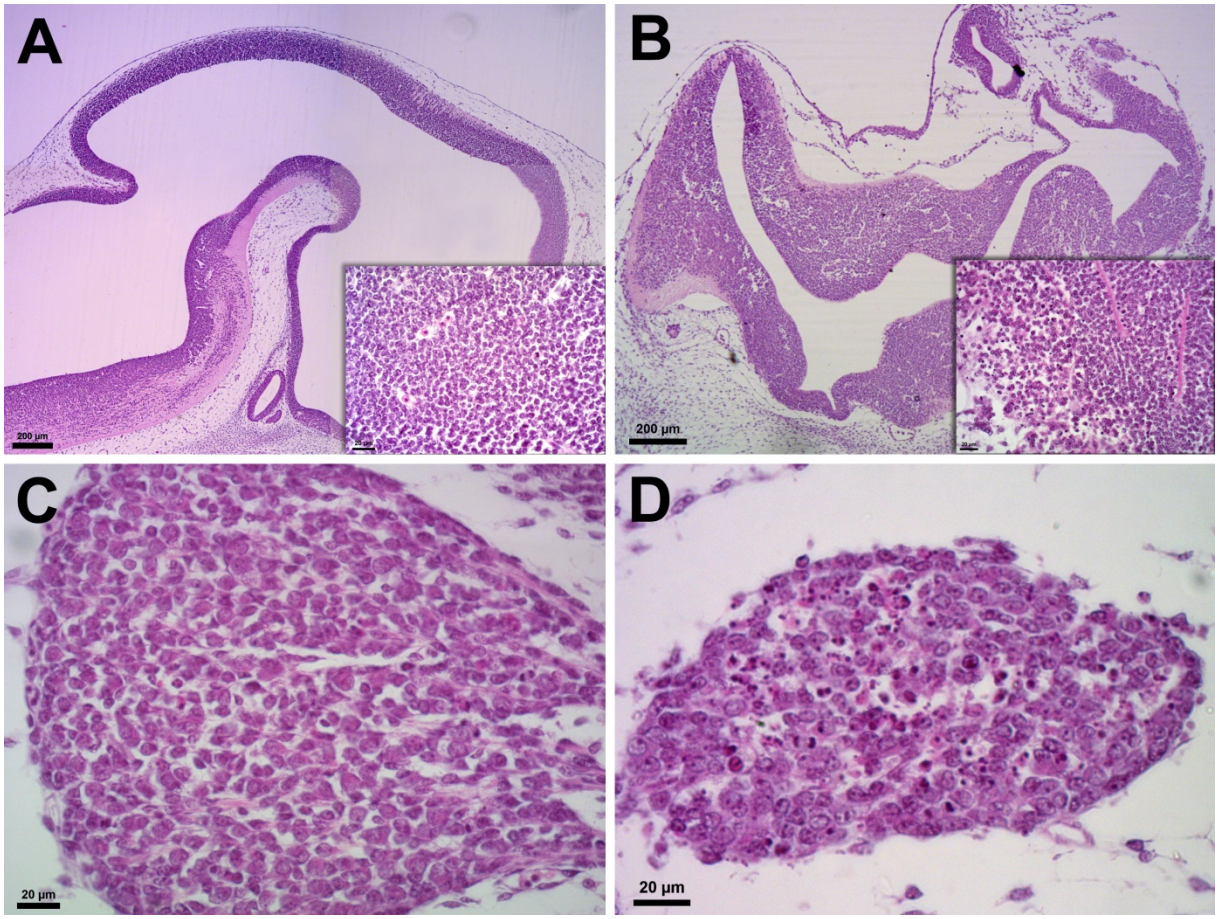

Figure 6. Representative alterations on central nervous system (CNS) and peripheral nervous system (PNS) in sagittal sections from mice embryos in GD 12 after *in utero* infection with *P. berghei* ANKA-GFP 10^6 parasite erythrocytes and treatment with artesunate. Control embryo showed normal shape and size of encephalon (A) with well-organized tissue (insert A) and normal cells in peripheral ganglia (C). Infected embryo showed a reduction in the size and alteration in the shape of encephalon (B). Embryo exposed *in utero* to artesunate 30 mg/kg showed apoptosis in CNS (insert B). Embryo from the group in which pregnant mice were infected and treated with artesunate 30 mg/kg showed apoptosis in the peripheral ganglia (D).

4. Discussion and conclusion

This study evaluated the effects of artesunate treatment in a malaria experimental model during pregnancy. Malaria infection caused a reduction on maternal body weight gain, an increase in maternal organs weights and embryolethality. The benefits of antimalarial treatment are clearly shown by ameliorating the effects of this infection during pregnancy.

Severe malaria, that frequently occurs due to *P. falciparum* parasites, is a multifactorial disease with many host organs affected, such as kidneys, lungs, liver and brain [4, 26-28]. The increase on maternal organs weight attributable to infection observed here may be correlated to severe malaria. The treatment with artesunate was effective by reducing malaria complication on kidneys, but this protection did not happen for liver weight.

It is known that malaria infection causes splenomegaly because of this organ involvement on the removal of parasitic erythrocytes [29]. The even greater enlargement of the spleen caused by artesunate is probably a consequence of the drug induction of pitting, a process of parasite clearance mediated by this organ. This mechanism is related to this drug fast mode of action, because mostly early parasite stages are rapidly removed through it [30].

The artesunate treatment did not cause maternal toxicity but at the highest dose caused embryo death and negatively affected the surviving embryos. This corroborates several reports about the developmental toxicity of artemisinin derivatives in animals [5, 6, 8-11]. The mechanism of embryotoxicity of these drugs was also confirmed here through a reduction on the amount of circulating erythroblasts (Figure 2b,d and 4f) and its consequences on embryo tissues or deaths [9].

Nevertheless, the developmental toxicity of artemisinin derivatives is until now a controversial debate. Our results come in agreement with the embryolethality, including higher incidence of total litter loss, related to artesunate treatment during the organogenesis period [5, 8]. However, until now it has never been proved in humans [4, 31]. Clinical data did not show any adverse effect for mother or fetus treated in the second and third trimester, but data from pregnant women exposed in

the first trimester are very limited and not enough to ensure their safety [4]. Moreover, the period of exposure to artesunate (usually 3-7 days in humans) could be not enough to induce embryo toxic effects, even if the treatment occurs in the estimated sensitive window for toxicity in humans (4-9 weeks of gestation) [7,10].

In view of these conflicting literature data, in this non-clinical study it was evaluated the embryotoxicity of artesunate in the presence of the infection. Our findings highlighted the benefits of the treatment on embryo survival. Both, the infection and the treatment with artesunate highest dose isolated caused total litter loss (Table 1). Of note, when these independent factors were put together there was no total litter loss and less embryo death on the remaining litters (Table 1). The mechanisms underlying this protection of embryolethality may involve the activation of artesunate and its higher concentration in parasitized erythrocytes, which means a kind of selective toxicity to parasites, and therefore alterations on the distribution of this drug to fetus [13].

There are clinical trials and experimental studies showing that malaria infection may alter the pharmacokinetics of antimalarial drugs [32-34]. Indeed, an experimental malaria model shows that metabolizing enzyme systems can be altered by the infection in non-pregnant animals [21]. The infection also alters hepatobiliary and placental transporters, which can be used by drugs, in pregnant mice [20]. These changes may alter the concentration of drug that reaches the embryo/fetus. In fact, it was demonstrated that the antimalarial quinine, which is the first option to treat malaria in the first trimester of pregnancy [4], was reduced on fetal tissues from infected females in comparison to uninfected [35].

Despite the artesunate treatment (30 mg/kg) given to infected mice somehow apparently protect embryos from death, it was still able to induce embryo toxic

effects. It demonstrates that artesunate reached the embryonic erythroblasts circulating cells and was able to damage them independent of the presence of the infection. However, considering that there was no total litter loss on the infected group treated with artesunate on this dose, it is likely that less drug or active drug reach the embryo.

Overall, our findings bring light to the safety of artemisinin derivatives use during the first trimester of pregnancy. This is the first work which evaluates the developmental toxicity of an artemisinin derivative treatment on infected pregnant mice. Here we show that the embryolethality of artesunate 30 mg/kg was reduced in infected animals, although there was still embryo toxic effects on the remaining embryos. These results show that the benefits of the treatment may overcome the risks, at least in animals.

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5.3.1 Material suplementar do artigo 3

5.3.1.1 Cálculo dos índices histopatológicos

Na análise histopatológica dos cortes sagitais dos embriões empregou-se um cálculo de alterações histológicas que foi adaptado de Bernet e colaboradores (1999). As alterações observadas foram agrupadas em seis padrões subdivididos em tipos de alterações, conforme descrito abaixo:

1- *Células sanguíneas de linhagem eritrocítica*: na idade em que os embriões foram avaliados, prevalecem os eritroblastos primitivos em sua circulação. Foi avaliada a quantidade de células presentes na maioria dos vasos sanguíneos, a sua estrutura celular (buscando alterações na sua forma e coloração, sendo consideradas como normais células de citoplasma abundante e eosinofílico com núcleo íntegro) e integridade celular (a apoptose foi caracterizada por células com núcleo fragmentado e fragmentos citoplasmáticos fortemente eosinofílicos).

2- *Coração*: foram avaliadas alterações anatômicas na sua forma, a espessura das paredes ventriculares e atriais (verificada pelo número médio de camadas celulares da parede do miocárdio), a organização histológica e integridade tecidual.

3- *Sistema nervoso central*: Foram verificadas alterações no tamanho e forma do encéfalo, integridade celular no tecido nervoso (encéfalo e medula espinhal) e organização histológica.

4- *Sistema Nervoso Periférico*: Foi verificada a integridade tecidual nos gânglios cranianos e espinhais.

5- *Fígado*: foram avaliados seu tamanho (avaliado considerando todos os cortes seriados em conjunto) e organização histológica.

6- *Outras alterações*: os tecidos ou estruturas não englobados nos padrões acima foram avaliados quanto à integridade e organização celular, tais como: mesênquima intermediário, somático, cefálico e esplâncnico, somitos, arcos faríngeos, túbulos e ductos mesonéfricos e derivados endodérmicos.

Para cada uma das alterações foi atribuído um escore de 0 a 4 de acordo com o grau de intensidade (considerou-se a área afetada e/ou número de focos). Foi calculado então o índice histopatológico para cada padrão (I_p). Este índice reflete o grau de dano no padrão e permite a comparação entre os grupos experimentais. O índice de cada padrão é dado pela equação abaixo:

$$I_p = \frac{\sum a \times w}{\sum n_{alt}} \times 10$$

Onde,

a = escore da alteração (0 a 4);

w = grau de importância da alteração em relação ao risco de morte do embrião (1 a 3). Foi considerado como 1 para a alteração de organização tecidual e como 3 para todas as demais alterações por considerá-las de maior importância patológica no desenvolvimento do embrião.

n_{alt} = número total de alterações avaliadas dentro de cada padrão (varia de acordo com o padrão).

O cálculo do índice histopatológico total (I_H) representa o índice total de lesões observadas em cada embrião analisado. Tal índice permite a comparação das alterações totais nos embriões observadas em grupos experimentais. Este índice foi obtido pela soma de todos os padrões:

$$I_{H = I_{RBC}} + I_{Heart} + I_{CNS} + I_{PNS} + I_{Liver} + I_{Others}$$

Onde,

I_{RBC} = índice obtido para o padrão de alterações nas células sanguíneas de linhagem eritrocítica.

I_{HEART} = índice obtido para o padrão de alterações no coração.

I_{CNS} = índice obtido para o padrão de alterações no sistema nervoso central.

I_{PNS}= índice obtido para o padrão de alterações no fígado.

I_{OTHERS}= índice obtido para o padrão de demais alterações no embrião.

6. DISCUSSÃO

Neste estudo o modelo de malária durante a gestação padronizado em camundongos suíços apresentou características da malária placentária em humanos, como: maior susceptibilidade das fêmeas à infecção durante a gestação, ocorrência de parto prematuro, alterações placentárias, fetos com baixo peso e com retardo no crescimento ósseo, caracterizando um retardo no desenvolvimento intrauterino (NERES *et al.*, 2008; HVIID *et al.*, 2010; RODRIGUES-DUARTE *et al.*, 2012). Tais resultados corroboram diversos trabalhos experimentais com outras linhagens de camundongos que vêm sendo utilizados para estudar a patogênese desta doença (NERES *et al.*, 2008; MARINHO *et al.*, 2009; RODRIGUES-DUARTE *et al.*, 2012; BARBOZA *et al.*, 2014).

Modelos experimentais de malária também são amplamente utilizados durante a fase pré-clínica do desenvolvimento de fármacos para avaliação de eficácia após testes *in vitro* (FLANNERY *et al.*, 2013). No entanto, tais modelos são pouco utilizados para estudos de toxicidade. Do mesmo modo, é pouco avaliada a eficácia/toxicidade de drogas antimaláricas em modelos de malária durante a gestação. Recentemente, Sharma e Shukla (2014) avaliaram a eficácia do tratamento preventivo com a sulfadoxina-pirimetamina em modelo experimental, verificando uma redução no estresse oxidativo induzido pela infecção no tecido placentário e um aumento na sobrevivência da prole devido ao tratamento.

O emprego de um modelo de malária experimental durante o período gestacional apresenta-se como uma ferramenta valiosa para estudos farmacológicos e toxicológicos. Algumas mudanças ocasionadas no organismo hospedeiro são reproduzidas nestes modelos animais e podem ser de grande relevância no perfil farmacocinético e toxicocinético das drogas, podendo alterar seus efeitos tóxicos sobre o desenvolvimento embrionário e fetal. De fato, um estudo demonstrou uma redução na concentração de quinina nos tecidos feto-placentários de animais expostos à infecção quando comparada à concentração encontrada em fetos de progenitoras não infectadas (LIRUSSI e PUSSARD, 2006).

Considerando a importância das alterações ocasionadas pela infecção no organismo hospedeiro, nós utilizamos o modelo previamente estabelecido para avaliar os efeitos embriotóxicos do artesunato, um derivado da artemisinina

amplamente utilizado. Nós evidenciamos a eficácia do tratamento na redução da parasitemia e na prevenção dos efeitos negativos da infecção sobre o ganho de peso materno, peso de órgãos maternos e na mortalidade embrionária.

Como mencionado anteriormente os derivados da artemisinina ainda não têm a segurança do seu uso estabelecida durante o primeiro trimestre gestacional. Isto se deve aos resultados controversos de estudos clínicos e pré-clínicos. Em animais eles são reconhecidos como toxicantes do desenvolvimento, causam morte embrionária e efeitos teratogênicos em doses terapêuticas e altas. No entanto, tais efeitos tóxicos ao embrião, até o momento, não foram comprovados em humanos (WHO, 2015b).

Os efeitos embriotóxicos do artesunato isolado na dose de 30 mg/kg foram confirmados neste trabalho, isto foi evidenciado pelo elevado percentual de perda total da ninhada (caracterizada por embriões mortos), aumento de perdas pós-implantes, redução do percentual de embriões vivos, saco embrionário marcadamente pálido e alterações histológicas nos embriões. Tais efeitos ratificam também o mecanismo de embriotoxicidade do artesunato através da redução de eritroblastos (LONGO *et al.*, 2006).

A mortalidade embrionária observada no grupo exposto à maior dose de artesunato foi reduzida pela presença da infeção. É importante notar que houve altos percentuais de perda total da ninhada tanto devido à infecção *per se,* quanto devido ao tratamento com o artesunato na maior dose. Este efeito de perda total da ninhada não foi verificado quando os animais foram infectados e posteriormente tratados com esta dose, demonstrando o benefício do tratamento na redução da embrioletalidade. No entanto os demais efeitos embriotóxicos, verificados pela redução de eritroblastos e pelos danos nos tecidos embrionários observados nos embriões vivos, foram similares para os grupos expostos ao artesunato na maior dose independentemente da infecção.

Um estudo pré-clínico reportou uma redução da toxicidade do artesunato em animais infectados quando comparado aos não infectados, verificada principalmente por danos renais em doses altas (LI *et al.*, 2007). Em humanos também foi demonstrada a redução dos efeitos tóxicos dos derivados da artemisinina aos reticulócitos, caracterizada por reticulocitopenia, em indivíduos com malária em relação aos não infectados (CLARK, 2012). Devido a isso, Clark (2012) levantou a

hipótese de que a mesma proteção poderia ocorrer para os efeitos embriotóxicos e isto foi confirmado neste trabalho no que se refere à embrioletalidade.

Esta redução da toxicidade devido à infecção, evidenciada em humanos ou animais, pode estar relacionada a algum grau de toxicidade seletiva dos derivados da artemisinina ao parasita. Isto é reforçado pela maior concentração da droga em eritrócitos infectados do que em não infectados observada *in vitro* (GU *et al.*, 1984), assim como por maiores concentrações destas drogas no plasma de pessoas infectadas quando comparadas a indivíduos saudáveis (CLARK, 2012).

Embora o mecanismo de ação dos derivados da artemisinina esteja pouco esclarecido, a sua ativação ocorre por mecanismos que envolvem o parasita e sua interação com diversas proteínas do mesmo podem influenciar em sua distribuição aos tecidos-alvo e, consequentemente, sua toxicidade (CLARK, 2012; WANG *et al.*, 2015). A ativação dos derivados da artemisinina pelo grupamento heme ou pelo íon ferro no estado ferroso envolve a presença e o metabolismo do parasita. O parasita digere a hemoglobina presente nos eritrócitos para se alimentar liberando o grupamento heme. Este grupamento heme livre pode catalisar a abertura da ponte peróxido o que faz com que seja gerada mais droga ativa para combater o parasita. O parasita também sintetiza o grupamento heme o que teria grande importância na ativação da droga nos estágios iniciais de desenvolvimento do mesmo (WANG *et al.*, 2015).

Desta maneira, é provável que durante a infecção a maior concentração de droga ativa no interior de eritrócitos parasitados (GU *et al.*, 1984) e ligada a proteínas do parasita (WANG *et al.*, 2015), resulte nas maiores concentrações plasmáticas observadas em pessoas com malária (CLARK, 2012; WANG *et al.*, 2015). Obviamente isto poderia alterar a distribuição da droga para os demais compartimentos, inclusive para o compartimento fetal. Os resultados aqui apresentados corroboram esta hipótese, possivelmente a concentração de artesunato que chegou aos embriões de progenitoras infectadas causando depleção de eritroblastos e dano aos tecidos embrionários tenha sido menor do que no grupo não infectado, ocasionando menor mortalidade embrionária.

Assim, um dos diferenciais entre estudos clínicos e pré-clínicos com os derivados da artemisinina na gestação pode ser a presença da infecção. Porém, estes resultados devem ser analisados com cautela quanto a sua extrapolação na

análise dos riscos relacionados à exposição humana. O protocolo da OMS em vigor preconiza o tratamento com artesunato no primeiro trimestre gestacional em casos de malária severa onde há risco de morte materna (WHO, 2015b). Para que seu uso seja permitido no primeiro trimestre gestacional ainda são necessários estudos clínicos com maior número de mulheres avaliadas.

No estudo aqui apresentado foi avaliado apenas o tratamento com o artesunato, enquanto que o tratamento com derivados da artemisinina atualmente é realizado por meio de ACTs. O artesunato é administrado isoladamente em casos de malária severa como uma dose de ataque até que o paciente tenha condições de ingerir ACTs por via oral (WHO, 2015b). Estudos pré-clínicos com ACTs em modelos experimentais, como o que aqui foi apresentado, poderiam fornecer informações importantes sobre a embriotoxicidade destas drogas.

Neste sentido, estudos pré-clínicos realizados pelo nosso grupo com a associação artesunato-mefloquina, evidenciaram uma redução dos efeitos tóxicos do artesunato sobre o desenvolvimento fetal quando estas drogas foram administradas concomitantemente (BOARETO *et al.*, 2012, 2013). Os mecanismos envolvidos nesta redução de toxicidade não foram elucidados, mas também podem ser afetados pela infecção e merecem ser investigados.

Contudo, os dados aqui apresentados reforçam hipóteses já levantadas anteriormente de uma redução na toxicidade dos derivados da artemisinina devido à infecção e provêm informações sobre a toxicidade do artesunato administrado no período da organogênese em camundongos. Também fica clara a importância das alterações ocasionadas pela infeção no organismo hospedeiro na avaliação de toxicidade pré-clínica de antimaláricos, e o modelo aqui estabelecido tem grande utilidade para estudos nesta área.

7. CONCLUSÃO

Neste estudo foram avaliados os efeitos embriotóxicos do artesunato em um modelo de malária estabelecido em camundongos suíços, para avaliação em conjunto das alterações causadas pela infecção e pelo tratamento.

A infecção com *P. berghei* mimetizou características observadas em casos de malária *falciparum* durante a gestação em humanos, como o baixo peso fetal, parto prematuro, alterações placentárias, mortalidade embrionária e retardo no desenvolvimento fetal. Foi demonstrado pela primeira vez um retardo no desenvolvimento ósseo nos fetos de fêmeas infectadas, caracterizando um retardo no crescimento intrauterino. Foi verificada a maior susceptibilidade das fêmeas a infecção devido à gestação, uma redução no ganho de peso materno e alterações no peso de órgãos maternos envolvidos na patogênese desta doença.

O tratamento com o artesunato na maior dose utilizada corroborou os efeitos embriotóxicos do artesunato descritos na literatura. No entanto, nesta dose foi observada uma redução na mortalidade embrionária (perda total da ninhada), causada tanto pelo artesunato quanto pela infecção isolados, quando esta droga foi administrada em progenitoras previamente infectadas. É possível que a concentração de artesunato que chegou aos embriões tenha sido reduzida pela presença dos parasitas resultando em menor embrioletalidade, embora ainda tenha sido suficiente para causar embriotoxicidade. Estudos complementares são necessários para comprovar os mecanismos envolvidos na redução de mortalidade embrionária observada neste trabalho, tais como a avaliação de distribuição da droga para o compartimento fetal. Também foram verificados os benefícios do tratamento com o artesunato sobre a toxicidade materna causada pela infecção e sua eficácia na redução da parasitemia.

Assim, alterações causadas pela própria infecção podem estar entre os fatores envolvidos nos resultados discrepantes de estudos clínicos e pré-clínicos sobre a segurança do uso dos derivados da artemisinina no primeiro trimestre gestacional. Contudo, os benefícios do artesunato no tratamento da infecção, neste modelo experimental, foram superiores aos riscos da infecção ou do tratamento isolados tanto para as progenitoras quanto para o embrião.

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